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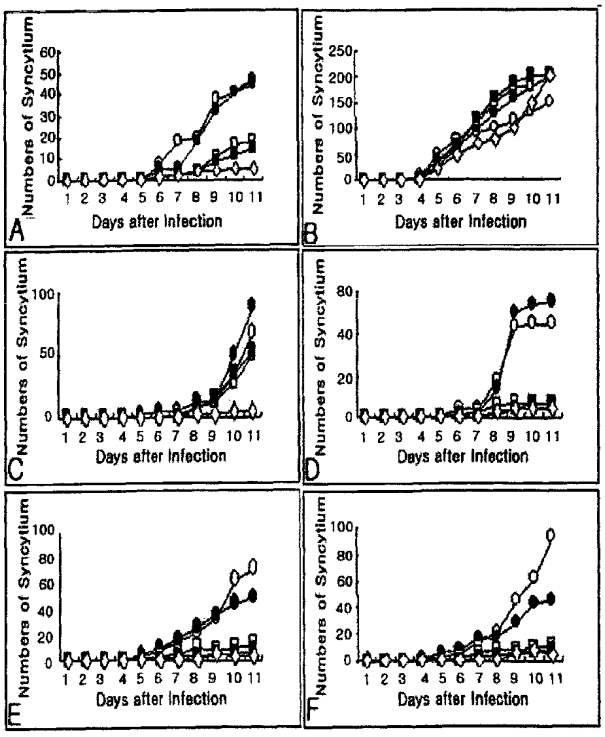
[Continued on next page]

(54) Title: PHOSPHOROTHIOATE (P=S) OLIGONUCLEOTIDES CONTAINING MODIFIED NUCLEOTIDES WITH SIX-MEMBERED AZASUGARS AND USE FOR AIDS THERAPY THEREOF



(57) Abstract: The present invention relates to a use of antisense oligonucleotides as an AIDS therapeutic agent. More precisely,the present invention relates to a novel use of antisense oligonucleotides as an AIDS therapeutic agent which include nucleotide derivatives in which five-membered ribose, a natural nucleotide sugar, is substituted with six-membered azasugar, and consisting of P=S backbone. Oligonucleotides of the present invention have low toxicity against cells and are working outside of cells to inhibit the viral attachment of HIV, with wide anti-viral activity against a broad spectrum of HIV variants. Therefore, oligonucleotides of the present invention can be used as one of effective AIDS therapeutic agent.

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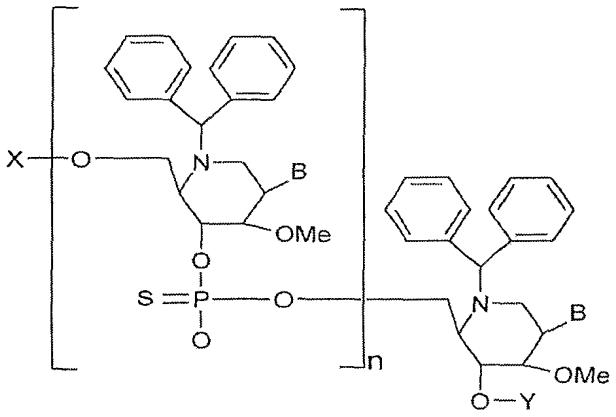
PHOSPHOROTHIOATE(P=S) OLIGONUCLEOTIDES CONTAINING MODIFIED NUCLEOTIDES WITH SIX-MEMBERED AZASUGARS AND USE FOR AIDS THERAPY THEREOF

FIELD OF THE INVENTION

The present invention relates to a use of synthetic oligonucleotides as an AIDS therapeutic agent. More precisely, the present invention relates to a novel use of specially designed synthetic oligonucleotides as an AIDS therapeutic agent which include nucleotide derivatives having the structure <Chemical Formula 1> shown below, in which five-membered ribose, a natural nucleotide sugar, is substituted with six-membered azasugar, and consisting of phosphorothioate (P=S) backbone. Oligonucleotides of the present invention have low toxicity against cells and are working outside of cells, resulting in inhibition of HIV-1 replication against a wide spectrum of HIV-1 variants by blocking the viral attachment. Therefore, oligonucleotides of the present invention can be used as one of effective AIDS therapeutic agents.

<Chemical Formula 1>



In the above <Chemical Formula 1>,

n = 0 ~ 30,

1) B is a wild-type or a modified nucleobase with or without a protecting group,

2) X is hydrogen, hydroxyl protecting group, conjugate group or oligonucleotide,

3) Y is hydrogen, phosphate, activated phosphate, activated phosphite, solid support, conjugate group or oligonucleotide.

BACKGROUND

The AIDS (acquired immunodeficiency syndrome) patient was first reported in Los Angeles, USA in 1982. Since then, 34,300,000 HIV carriers (Global summary of the HIV/AIDS epidemic, end 1999) have been reported to WHO by the end of 1999. In Korea, since the first American AIDS patient was reported in 1985, the numbers of patients have been steadily increased, with an

estimation of 1,173 HIV carriers as of June, 2000(included 186 AIDS patients, Communicable Disease Monthly Report, July issue of 2000. Korean NIH), which caused serious debate on AIDS therapy. Because of the rapidly growing number of HIV carriers and AIDS patients, USA, France, Canada, Japan and other countries have studied to develop AIDS therapeutic agents. 2,3-azidothymidine (AZT), dideoxyinosine (ddI) and dideoxycytidine (ddC) which have been approved by FDA (USA) are known as anti-HIV agents so far. However, those agents have their own limitations to a curative effect as well as having serious side effects. Thus, it is very important to develop a novel AIDS therapeutic agent.

HIV belongs to the Retrovirus Family, which keeps genetic information as a form of RNA. Concerning the structure of the virus particle, viral RNA genome and reverse transcriptase are encapsulated by capsid protein, and lipid membrane is covering the nucleocapsid particle. Glycoproteins, gp120 and gp41, are located in the lipid membrane and especially, gp120 is necessary for virus attachment to T-cells and penetration into thereafter.

For the treatment of AIDS, various types of therapeutic agents have been tried but not yet been fully successful since HIV virus has the most

complicated replication mechanism. Reverse transcriptase inhibitor is known to be the most effective one so far. Reverse transcriptase is essentially required for synthesis of provirus DNA from the viral RNA genome. Owing to the above characteristics, reverse transcriptase inhibitors have been studied to disrupt HIV-1 replication. Azidothymidine (Glaxo-Wellcome), 2',3'-dideoxyinosine (Bristol Myers-Squibb), 2',3'-dideoxycytidine (Hoffmann-La Roche), D4T (Bristol Myers-Squibb) and 3TC (Glaxo-Wellcome) have been developed and clinically used as reverse transcriptase inhibitors. Even though those compounds are efficient to inhibit the HIV-1 replication in vitro, they are not so effective in vivo as shown in vitro, resulting in rather prolonging survival time of patients than curing the disease completely. Those compounds also cause serious side effects such as degeneration of platelets and blood stem cells. Besides, many variants showing resistance to those compounds have been pointed out to be another problem.

In order to overcome those problems, another crucial therapeutic agent, HIV protease inhibitor, has been developed. HIV does not synthesize capsid protein and enzyme, respectively, from mRNA, but produce long polyprotein first, such as gag-protein (p55) or gag-pol

protein (p165), in which capsid proteins and enzymes are all integrated. The capsid proteins, reverse transcriptase and integrase are cleaved by HIV-1 protease residing in the long polyprotein. Thus, protease inhibitor has been developed on the basis of the idea that the HIV-1 replication will be quenched if viral protease responsible for polyprotein processing is inhibited. HIV protease consists of two identical monomers (homodimer) each of which has 99 amino acids (molecular weight of each monomer is 10,793 daltons). HIV protease is a typical aspartic acid protease characterized by the sequence of aspartate-threonine-glycine on an active site.

As demonstrated in the process of developing inhibitors (especially lenine) against other aspartic acid proteases, HIV protease inhibitors has been developed from selecting the compounds showing high affinity to the viral protease by simulating the transition state of enzyme to form the transition state analogue (TSA). HIV protease inhibitors developed on the basis of that concept have been reported (Robert, et al., *Science*, 248, 358, 1990; Europe patent application #0337714; #0346847; #356223; #352000; #357332; #362002; #361341; Bone, et al., *JACS*, 113, 9382, 1991).

Another promising approach to AIDS treatment is to

use antisense oligonucleotides, which can be hybridized with messenger RNA specifically. For protein synthesis, only one strand of DNA double helix is transcribed into mRNA, which is later translated into protein in the cytoplasm. Unlike other therapeutic agents, the antisense oligonucleotides are targeting mRNA to block the translation of mRNA into proteins. The mechanism of antisense oligonucleotides is to cut off the target mRNA or disrupt the ribosome-mediated translation of mRNA into protein, resulting in the lack of the target protein synthesis. At this time, oligonucleotides are complementary to the target mRNA sequence, so that the oligonucleotide is named by antisense oligonucleotide and the technique is called antisense technology.

In the late 1970s, synthetic DNA fragment has been reported to be effective to inhibit the viral protein synthesis by Stephenson and Zamecnik (Stephenson, et al., *Proc. Natl. Acad. Sci. USA*, 95, 285, 1977; Zamecnik, et al., *proc. Natl. Acad. Sci. USA*, 95, 280, 1977). In 1980s, it was also disclosed that antisense RNA spontaneously synthesized in the organisms regulates its own gene expression in the bio-system (Simons, et al., *Cell*, 34, 683, 1983; Mizuno, et al., *Proc. Natl. Acad. USA*, 81, 1966, 1984).

Even though efficient in vitro, the natural

oligonucleotides themselves are easily decomposed by nucleases *in vivo*. So that it is hardly expected that those oligonucleotides have sufficient pharmacological effect in vivo. Intensive efforts have been concentrated for generating more stable antisense compounds by modification of the structure of antisense oligonucleotides.

Oligonucleotides having P=S or methylphosphate backbone instead of P=O bonding, have been developed as a first generation of antisense agents. The P=S oligonucleotide which has a sulfur instead of oxygen in phosphate group of the P=O backbone, has lower affinity to mRNA than natural oligonucleotide does, but has a strong resistance to nucleases and an effective pharmacological activity not only in vitro but also in vivo. Some of the antisense agents of first generation are in the midst of clinical test as anti-viral or anti-cancer agents, and some have already been into the market as anti-viral agents (Bennett, et al., *Biochem. Pharmacol.*, 55, 9-19, 1998). However, those still have such limitations as toxicity or unwanted immune responses (Stein, et al., *Current Opinion in Oncology*, 6, 587-594, 1994; Krieg, et al., *Nature*, 374, 549, 1995; O'brien, et al., *leukemia*, 8, 2156, 1994). In order to overcome the shortcomings mentioned above, several different approaches such as modification of

phosphate backbones of oligonucleotides with amide or ether, changes of base structures of oligonucleotides, changes of ribose structures, etc., have been made (De Mesmaeker, et al., *Acc. Chem. Res.*, 28, 366-374, 1995).

While oligonucleotides whose phosphate backbone was changed such as P=S, methylphosphate, etc. are called the first generation antisense molecules, second-generation antisense oligonucleotides have a modified sugar. Oligonucleotides having methoxy, methoxyethoxy (Martin, et al., *Helv. Chim. Acta.*, 186, 584, 1995) or aminoalcoxy group (Griffey, et al., *J. Med. Chem.*, 39, 5100-5109, 1996) introduced to 2′ of ribose, oligonucleotides containing hexose (Herdewijn, et al., In Carbohydrate Modifications in Antisense Research, ACS Symposium Series 580; Sanghvi, Y. S., Cook, P. D., Eds., American Chemical Society, Washington, DC, pp 80-99, 1994), oligonucleotides containing pentose (Moser, et al., Strategies and Chemical Approaches toward Oligonucleotide Therapeutics. In Perspectives in Medicinal Chemistry; Testa, B., et al., Eds.; Verlag Helvetica Chimica Acta: Basel, pp 275-97, 1993), oligonucleotides containing 4′-aminoribose(Scharer, et al., *J. Am. Chem. Soc.*, 117, 6623-6624, 1995), oligonucleotides containing 4′-thioribose(Bellon, et al., 4-Thio RNA: a novel class of sugar-modified B-RNA. In Carbohydrate Modifications in

Antisense research; ACS Symposium Series 580; Sanghvi, Y. S., Cook, P. D., Eds.; American Chemical Society: Washington, DC, 1994; pp 68-79) and their derivatives are the examples for the second generation antisense molecules.

It was confirmed by measuring UV absorbance that mRNA and DNA existed as duplex at room temperature (or body temperature), but once the temperature rose, the duplex was separated into single strands. UV absorbance of a single strand is higher than that of duplex, that is to say, if the amount of single strand increases by temperature rise, UV absorbance also increases resulting in sigmoid changing curve. The temperature giving middle level of UV absorbance in the sigmoid curve is defined as a Tm (melting temperature). High Tm to mRNA means high affinity (binding capacity) of oligonucleotides to RNA, which is a very important factor as an effective antisense molecule. Several groups have measured the affinity of oligonucleotides having various substitutions introduced to 2′ site of RNA (Breslauer, et al., *Proc. Natl. Acad. Sci. USA*, 83, 3740, 1986; Freier, et al., *Nucleic Acids* Res., 25, 4429-4443, 1997).

Among the second-generation antisense molecules, oligonucleotides with the modified base having methoxy

group at 2'-site of ribose showed rather high Tm to mRNA, which was likely due to that the introduced electronegative groups such as methoxy and fluorine increased the affinity to RNA (Kawasaki, et al., *J. med. Chem.*, 36, 831-841, 1993). And also oligonucleotides having alcoxy group such as methoxy at 2'-site showed increased resistance against nucleases comparing to natural DNA. 2'-methoxy substituent and P=S form chimeric oligonucleotides which have less toxicity in comparison with to the first generation antisense oligonucleotides (Lesnik, et al., *Biochemistry*, 32, 7832, 1993; Milligan, et al., *J. Med. Chem.*, 36, 1923, 1993), and their clinical trials started in 1997(Agrawal, et al., *Curr. Opin. Chem. Biol.*, 2, 519, 1988).

The present inventors have studied to develop antisense oligonucleotides which are more stable and resistant against nucleases and also have higher affinity to mRNA. As a result, it has been developed and confirmed that newly designed oligonucleotides containing 6-membered azasugars instead of 5-membered ribose have greater affinity and stability, which were filed for a patent (Korea patent application #99-26947).

The present invention has been accomplished by verifying that oligonucleotides of the present

invention can be effectively used as an AIDS therapeutic agent since unlike the conventional antisense, oligonucleotides of the present invention containing 6-membered azasugars and consisting of P=S backbone show potent antiviral activity against a broad spectrum of HIV variants as well as low cytotoxicity.

SUMMARY OF THE INVENTION

It is an object of this invention to provide oligonucleotides with P=S backbone, which contain nucleotide derivatives whose five-membered ribose, a natural nucleotide sugar was substituted with six-membered azasugar.

It is another object of this invention to provide a therapeutic agent containing the above oligonucleotides for the treatment of AIDS.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is photographs observed by light microscopy showing the antiviral activity of oligonucleotides of the present invention against HIV-1 replication. They are showing the cytopathic effect observed on 7^{th} day after virus infection,

A: Control,

B: Oligonucleotide of the SEQ. ID. NO: 2,

C: Oligonucleotide of the SEQ. ID. NO: 4,

D: Oligonucleotide of the SEQ. ID. NO: 6,

E: Oligonucleotide of the SEQ. ID. NO: 11,

F: Oligonucleotide of the SEQ. ID. NO: 13,

G: Oligonucleotide of the SEQ. ID. NO: 17,

H: Oligonucleotide of the SEQ. ID. NO: 22

FIG. 2 is graphs showing the antiviral activity of oligonucleotides of the present invention against HIV-1 replication with varying concentrations in a serum-free medium.

A: Oligonucleotide of the SEQ. ID. NO: 2,

B: Oligonucleotide of the SEQ. ID. NO: 4,

C: Oligonucleotide of the SEQ. ID. NO: 11,

D: Oligonucleotide of the SEQ. ID. NO: 17,

E: Oligonucleotide of the SEQ. ID. NO: 22,

F: Oligonucleotide of the SEQ. ID. NO: 29,

○ : 0.02 μM, ● : 0.04 μM, □ : 0.06 μM,

■ : 0.08 μM, ◇ : 0.1 μM

FIG. 3 is a graph showing the antiviral effect of oligonucleotides of the SEQ. ID. NO: 2 and 22 of the present invention, on HIV-1 replication in serum-free Opti-MEM medium by measuring the reverse-transcriptase activity of HIV-1.

○ : Control,

● : Oligonucleotide of the SEQ. ID. NO: 2,

■ : Oligonucleotide of the SEQ. ID. NO: 22

FIG. 4 is a graph showing the antiviral effect of oligonucleotides of the present invention on HIV-1 replication in RPMI 1640 medium supplemented with 10% fetal bovine serum by measuring the reverse-transcriptase activity of HIV-1.

0 : Control,

□ : Oligonucleotide of the SEQ. ID. NO: 2,

● : Oligonucleotide of the SEQ. ID. NO: 4,

◇ : Oligonucleotide of the SEQ. ID. NO: 17,

△ : Oligonucleotide of the SEQ. ID. NO: 20,

■ : Oligonucleotide of the SEQ. ID. NO: 22

FIG. 5 is photographs showing the short-term (hourly) and the long-term (daily) stability of oligonucleotides of the present invention against nuclease degradation in the supernatant of cell culture medium.

A: Oligonucleotide of the SEQ. ID. NO: 2,

B: Oligonucleotide of the SEQ. ID. NO: 4,

C: Oligonucleotide of the SEQ. ID. NO: 5,

D: Oligonucleotide of the SEQ. ID. NO: 13

FIG. 6 is photographs showing the long-term stability of oligonucleotides of the present invention

against nuclease degradation in the fresh RPMI 1640 medium supplemented with 10% fetal bovine serum.

A: Oligonucleotide of the SEQ. ID. NO: 2,

B: Oligonucleotide of the SEQ. ID. NO: 22

FIG. 7 is photographs of the experimental results showing whether the oligonucleotides of the SEQ. ID. NO: 2, 4 and 22 of the present invention are able to inhibit HIV-1 LTR activity in Magi cells (HeLa-CD4 cells containing LTR-β-gal gene) when they are simply added in the culture medium or when they are transfected into the cells.

A: 2 μg of pSV2-Tat was transfected into Magi cells, after which 0.25 μ M of oligonucleotides were simply added into the medium,

B: 2 μg of pSV2-Tat and 2 μg of oligonucleotides were co-transfected into Magi cells

FIG. 8 is the graph showing the inhibition capacity of oligonucleotides of the present invention against the HIV-1 LTR activity when they were transfected into the cells in different concentrations.

◇ : Oligonucleotide of the SEQ. ID. NO: 2,

■ : Oligonucleotide of the SEQ. ID. NO: 4,

0 : Oligonucleotide represented by the SEQ. ID. NO: 17,

□ : Oligonucleotide of the SEQ. ID. NO: 22

FIG. 9 is a photograph showing the result of CAT assay in the LTR-CAT-transfected Jurkat-tat cells, which was performed to see whether the oligonucleotides of the SEQ. ID. NO: 2 and 4 were able to pass through cell membrane and inhibit the LTR function in nuclei when they are simply added into the culture medium.

A: Control (LTR-CAT was not transfected),

B: Control(cells were transfected with 5 μg of LTR-CAT without further treatment of oligonucleotide),

C: Cells were transfected with 5 μg of LTR-CAT, and then treated with the oligonucleotide of the SEQ. ID. NO: 2,

D: Cells were transfected with 5 μg of LTR-CAT, and then treated with the oligonucleotide of the SEQ. ID. NO: 4

FIG. 10 is a photograph showing the result of CAT assay, which was performed to see if the oligonucleotides of the present invention have sequence-specific anti-viral activity against HIV-1 LTR within the Jurkat-tat cells when they were co-transfected into the cells with LTR-CAT plasmid DNA,

A: Control(cells transfected with 5 μg of LTR-CAT),

B: cells co-transfected with 5 μg of LTR-CAT and 2 μg of oligonucleotide SEQ. ID. NO: 2,

C: cells co-transfected with 5 μg of LTR-CAT and 2

μg of oligonucleotide SEQ. ID. NO: 4,

D: cells co-transfected with 5 μg of LTR-CAT and 2 μg of oligonucleotide SEQ. ID. NO: 9,

E: cells co-transfected with 5 μg of LTR-CAT and 2 μg of oligonucleotide SEQ. ID. NO: 11,

F: cells co-transfected with 5 μg of LTR-CAT and 2 μg of oligonucleotide SEQ. ID. NO: 12,

G: cells co-transfected with 5 μg of LTR-CAT and 2 μg of oligonucleotide SEQ. ID. NO: 13

FIG. 11 is photographs showing the curing effects of the oligonucleotides of the present invention on the HIV-1-infected Jurkat cells when large amounts of syncytia (giant cells especially appeared in HIV-1 infected cells) were already produced,

A: Normal Jurkat cells,

B: Jurkat cells, 5 days after HIV-1 infection,

C: Jurkat cells, 7 days after HIV-1 infection,

D: 36 hours after treating C with oligonucleotide of the SEQ. ID. NO: 2,

E: 36 hours after treating C with oligonucleotide of the SEQ. ID. NO: 4,

F: 36 hours after treating C with oligonucleotide of the SEQ. ID. NO: 17,

G: 36 hours after treating C with oligonucleotide of the SEQ. ID. NO: 20,

H: 36 hours after treating C with oligonucleotide

of the SEQ. ID. NO: 22

FIG. 12 is a graph showing the effect of the oligonucleotides of the present invention on the SIV replication,

○ : Control,

□ : Oligonucleotide of the SEQ. ID. NO: 6,

■ : Oligonucleotide of the SEQ. ID. NO: 7,

◇ : Oligonucleotide of the SEQ. ID. NO: 2,

◆ : Oligonucleotide of the SEQ. ID. NO: 4,

△ : Oligonucleotide of the SEQ. ID. NO: 13,

● : Oligonucleotide of the SEQ. ID. NO: 22

FIG. 13 is a graph showing the effect of the oligonucleotides of the present invention on the poliovirus Sabin 1 proliferation.

■ : control,

□ : Oligonucleotide of the SEQ. ID. NO: 2,

● : Oligonucleotide of the SEQ. ID. NO: 28

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

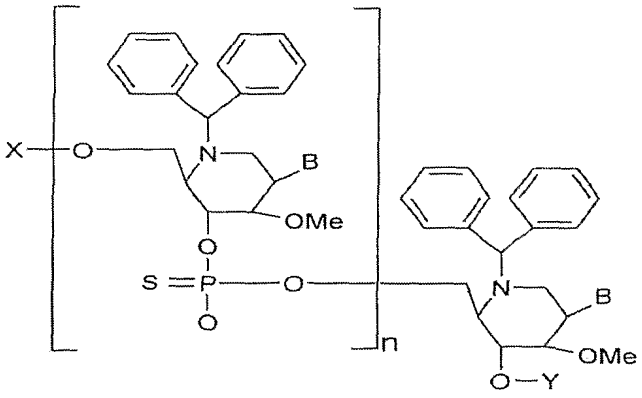
To accomplish those objects, the present invention provides oligonucleotides with P=S backbone, which contain nucleotide derivatives whose five-membered ribose, a natural nucleotide sugar was substituted with six-membered azasugar.

The present invention also provides a therapeutic agent used for the treatment of AIDS containing the above oligonucleotides.

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides oligonucleotides having the structure <Chemical Formula 1> shown below containing nucleotide derivatives whose five-membered ribose, a natural nucleotide sugar was substituted with six-membered azasugar, and consisting of P=S backbone.

<Chemical Formula 1>



In the above <Chemical Formula 1>,

n = 0 ~ 30,

1) B is a wild-type or a modified nucleobase with or

without a protecting group,

2) X is hydrogen, hydroxyl protecting group, conjugate group or oligonucleotide,

3) Y is hydrogen, phosphate, activated phosphate, activated phosphite, solid support, conjugate group or oligonucleotide.

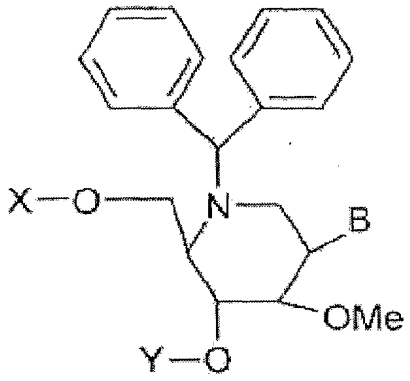
The range of n is 0~30 including nucleotides of both up and down position in the above <Chemical Formula 1>, and 6~21 is more preferable. The modified monomer of the present invention could be located in any position of oligonucleotides, and oligonucleotides having more than 4 modified monomers are preferable for the use in the treatment of AIDS. Oligonucleotides of the SEQ. ID. NO: 1~29 are preferable for the treatment of AIDS and further those of the SEQ. ID. NO: 2, 3, 11, 17, 18, 19, 20, 21, 22, 23 and 29 are more preferable.

Oligonucleotides of the present invention can be prepared in the condition of both liquid and solid phase, but the later is more preferable. The detailed synthetic method of oligonucleotides in the solid phase condition is described in Oligonucleotide Synthesis, A Practical Approach, Gait (ed.), IRL Press, Washington D. C. (1984), Caruthers, et al., U. S. Pat. No. 4,458,066 and 4,500,707.

Oligonucleotides of the present invention have

been prepared by condensation reaction using nucleotide monomer of the <Chemical Formula 2>(Korea patent application #99-26947) shown below. When being prepared, the primary alcohol group bound to nucleotide sugar should be replaced by dimethoxytrityl group and so does the secondary alcohol group by phosphoamidite group. Beside of thymine, nucleobases should be protected by the proper protecting group as well.

<Chemical Formula 2>



In the above <Chemical Formula 2>

1) B is a natural nucleobase or modified nucleobase with or without a protecting group,

2) X is hydrogen or hydroxyl protecting group,

3) Y is hydrogen, phosphate, activated phosphate, activated phosphite or solid support.

When nucleotide monomer of the <Chemical Formula 2> shown above is introduced to the 3'-site of oligonucleotides, this derivative needs to be attached

to solid support. This monomer was converted to hemiccinate by using the commercially available CPG (controlled pore glass) containing amino group, which was followed by condensation reaction under mesitylene-2-sulfonylchloride/1-methyl-1H-imidazole.

The monomer of <Chemical Formula 2> was introduced to any position except 3'-end of oligonucleotides by standard phosphoamidite method using DNA synthesizer (ex. ABI 392 etc.). In general, the concentration of monomer and its reaction timing with solid support were identical with those in phosphoamidite method using DNA synthesizer, but it was preferable to prolong the condensation time from 60 seconds to 600 seconds when hydrogen was replaced with other functional groups at 2'-site.

Once oligonucleotides are prepared, they should be separated from solid support and protecting group also should be removed. These procedures could be performed simultaneously or one by one. Once the synthesis of oligonucleotides are complete, they are released from solid support by treating ammonia water at room temperature, and the protecting group is removed by using ammonia water while being heated (generally at 55℃ for 17 hours). Dimethoxytrityl group, a protecting group for 5'-hydroxyl group of oligonucleotides is removed at the last step of synthesis using the program installed in DNA

synthesizer as well as by an additional treatment with 80% acetic acid, dichloroacetic acid or trichloroacetic acid.

In case of monomer having alcohol group at 2'-site, isobutyl group is used as a protecting group for the synthesis of oligonucleotides, and then removed by treatment with ammonia solution as used in general DNA synthesis protocol. Monomer having hydrogen at the nitrogen site of azarsugar is protected by fluorenyl group (F-moc), and is also separated from the protecting group after synthesis of oligonucleotides as described in the general protocols for separation of protecting group.

The preparation method of the oligonucleotides of the present invention with OF backbone, which contain nucleotide derivative having the structure of <Chemical Formula 1> was simplified by attaching nucleobase to 3'-carbon site of 6-membered azasugar (piperidine). When the base was attached to aminal site, nucleobases could be introduced to both alpha and beta position, which required an additional step for separation of isomers. However, the present inventors introduced a base to carbon site instead of aminal site, which resulted in nucleotides having a base only at beta position. In addition, many functional groups were easily introduced to nitrogen position using azasugar

instead of the tedious method of using strong base. Since the oligonucleotides having various groups introduced to nitrogen position show relatively high Tm against RNA, they can be effectively used as antisense agents having strong affinity to mRNA.

By introducing six-membered azasugar instead of ribose and attaching hydrophobic group to azasugar, the oligonucleotides of the present invention can improve the cell membrane permeability. Oligonucleotides containing carbocyclic nucleotides are known to be stable with strong resistance against enzymes and actually those of the present invention were confirmed to be highly resistant against nucleases. The enhanced affinity and stability to nucleases of the specially designed synthetic oligonucleotides of the present invention, at least in part, resulted from changing their P=O backbones into P=S backbone partially or as a whole.

Oligonucleotides of the present invention can be prepared in the form of chimeric oligonucleotides containing P=O or P=S in the oligonucleotide backbone.

The present invention also provides a pharmaceutical composition used for the treatment AIDS containing oligonucleotides of the present invention as an effective ingredient.

In order to verify the usability of synthetic oligonucleotides as a therapeutic agent used for treatment of AIDS, the present inventors have prepared oligonucleotides composing, entirely or in parts, 6-membered azarsugar and P=S backbone, whose sequences are complementary to various target sequences such as HIV-1 TAR, SIV (simian immunodeficiency virus) TAR, poliovirus IRES (internal ribosomal entry site), HIV splicing doner (sd), LTR, gag, random sequences, etc (see Table 1).

Anti-viral activity of oligonucleotides of the present invention against HIV-1 replication was measured by syncytium forming experiment. As a result, oligonucleotides of the present invention are superior in anti-viral activity to natural antisense oligonucleotide with P=O backbone (SEQ. ID. NO: 13) or to oligonucleotides with P=S backbone only (SEQ. ID. NO: 4) (see Fig. 1, 2, Table 2 and 4). Also, most of the oligonucleotides of the present invention (SEQ. ID. NOs: 2, 3, 11, 17-23 and 29) are superior to AZT (conventional AIDS therapeutic agent) showing long-lasting anti-viral activity for 12 days after a single treatment when tested in the same concentration (see Table 2 and 3).

Anti-HIV-1 activity of the oligonucleotides of the present invention was also examined by measuring reverse transcriptase activity in the HIV-1 infected

cells cultured in their presence. It was confirmed therefrom that virus replication was completely inhibited at 0.5uM of oligonucleotides regardless of the serum presence in medium (see Fig. 3 and 4). Oligonucleotides of the present invention were proved to have similar cytotoxicity to that of natural oligonucleotides (P=O bonds)(see Table 8), but have much potent anti-viral activity, which is expected to contribute to the safe treatment for the disease.

In order to investigate the stability against nuclease, oligonucleotides of the present invention were added into a culture supernatant (see Fig. 5) or a fresh medium (see Fig. 6) for an indicated period of time, and then harvested and examined for their integrity on polyacrylamide gel electrophoresis. As a result, oligonucleotide with P=O backbone having 6-membered azarsugar as well as oligonucleotides with P=S backbone without 6-membered azasugar have kept their stability without being decomposed even in cell culture supernatant over 6 days. On the contrary, oligonucleotides with P=O backbone only were decomposed completely within 2 hours (see Fig. 5). It means that the 6-membered azarsugar is also likely to contribute to the stability of the oligonucleotides of the present invention. Actually, oligonucleotides of the present invention kept their stability up to 15 days without

being decomposed in the serum-containing fresh medium (see Fig. 6).

In order to see whether oligonucleotides of the present invention are able to pass through cell membrane and have anti-viral activity within the cells, the expression of the HIV-1 LTR-mediated reporter gene was studied in cell culture in the presence of each oligonuceleotide. Particularly, cells containing genes for LTR-CAT or LTR-β -galactosidase, as a reporter system, were used for these experiments. Magi cells having LTR-β -galactosidase were treated or transfected with oligonucleotides of the present invention in order to examine their membrane permeability by measuring the inhibition level of HIV-1 LTR-mediated reporter expression. As a result, the number of cells expressing LTR-mediated β -galactosidase was not reduced when the cells were only treated with oligonucleotides of the present invention in the culture medium (see Fig. 7A). On the other hand, the number was markedly reduced in a sequence specific manner by transfecting cells with P=O oligonucleotide of the SEQ. ID. NO: 4 (see Fig. 7B). The inhibitory effect of the oligonucleotide of the SEQ. ID. 4 was proportional to the amounts of transfected oligonucleotide, but in cases of other P=S oligonucleotides, the amounts of oligonucleotides used

for transfection did not affect on LTR inhibition (see Fig. 8). In addition, while LTR-CAT gene-transfected cells being cultured in the presence of oligonucleotides of the present invention, did not affect on CAT expression controlled by LTR, (see Fig. 9), cells transfected with each oligonucleotide together with LTR-CAT plasmid showed sequence-specific inhibition strongly (SEQ. ID. NO: 4) or weakly (SEQ. ID. NO: 9 and 13) in CAT gene expression (see Fig. 10), resulting in good agreement with the previous result. However, oligonucleotides demonstrating high anti-HIV-1 activity did not inhibite the LTR-mediated CAT expression even when they were transfected into the cells.

These results suggest that the oligonucleotides of present invention containing 6-membered azasugar nucleotide with P=S backbone have an anti-HIV-1 activity whose mechanism is different from that of conventional antisense oligomers working in a sequence-specific manner. In addition, the inhibitory effect of a certain oligonucleotide (represented by the SEQ. ID. NO: 4) against LTR activity was largely increased only when being transfected into cells artificially, but did not even occurred when simply being added into medium, suggesting that the membrane permeability of the oligonucleotides of the present invention seems to be

very low when simply treated to culture media. Nevertheless, oligonucleotides still showed potent anti-viral activity regardless of membrane permeability and sequence-specificity, which led us to assume that they might be working outside of the cells probably by blocking viral attachment, resulting in effective shut off of the HIV-1 infection.

In order to test the usability of oligonucleotides of the present invention for the treatment of AIDS, they were added to HIV-infected cells which showed progressed cytopathy (lots of syncytia were observed), and then the change of cytopathy was observed. As a result, the number and the size of syncytia were remarkably reduced in the presence of oligonucleotides of the present invention in the culture. However, comparing to control group much more severe and larger syncytia were formed when oligonucleotide of P=S backbone with no 6-membered azarsugar nucleotide (represented by the SEQ. ID. NO: 4) was added (see Fig. 11). These results strongly suggest that oligonucleotides of the present invention can be used as a successful therapeutic agent against AIDS even for the highly advanced AIDS patients.

In order to see whether oligonucleotides of the present invention affect the replication of other

viruses than HIV-1 by blocking nonspecific cell surface molecules, SIV and poliovirus were tested for their replication in the presence of these oligonucleotides. Oligonucleotides of the present invention did not inhibit SIV proliferation regardless of the sequence-specificity to SIV TAR (see Fig. 12). In addition, poliovirus replication was also undisturbed by any oligonucleotides of the present invention having complementary to IRES (internal ribosomal entry site) or random sequence (see Fig. 13). These results strongly suggest that the oligonucleotides of the present invention specifically inhibit HIV-1 replication without influence on those of other viruses.

Serum effects on anti-viral activity of the oligonucleotides of the present invention were tested. Oligonucleotides of the present invention were added into media containing different kinds and different concentrations of sera, and the syncytium formation was observed therefrom. Many of the oligonucleotides of the present invention showed rather reduced anti-viral activity in the presence of serum. However, anti-viral activity of a specific oligonucleotide (represented by the SEQ. ID. NO: 22) was not clearly disturbed by serum addition (see Table 5).

Oligonucleotides of the present invention can be

administered orally or parenterally. The compounds of the present invention can be prepared for oral or parenteral administration by mixing with generally-used fillers, extenders, binders, wetting agents, disintegrating agents, diluents such as surfactant, or excipients. The present invention also includes pharmaceutical formulations in dosage units. This means that the formulations are presented in the form of individual parts, for example tablets, coated tablets, capsules, pills, suppositories and ampules, the active compound content of which corresponds to a fraction or a multiple of an individual dose. The dosage units can contain, for example, 1, 2, 3 or 4 individual doses or 1/2, 1/3 or 1/4 of an individual dose. An individual dose preferably contains certain amount of active compound, which is administered in one application and which usually corresponds to a whole, one half, one third, or a quarter of a daily dose. Non-toxic inert pharmaceutically suitable excipients are to be understood as solid, semi-solid or liquid diluents, fillers and formulation auxiliaries of all types. Preferred pharmaceutical formulations which may be mentioned are tablets, coated tablets, capsules, pills, granules, suppositories, solutions, suspensions and emulsions, pastes, ointments, gels, creams, lotions, dusting powders and sprays. Solid formulations for oral administration are tablets, pill, dusting powders

and capsules. Liquid formulations for oral administrations are suspensions, solutions, emulsions and syrups, and the abovementioned formulations can contain various excipients such as wetting agents, sweeteners, aromatics and preservatives in addition to generally used simple diluents such as water and liquid paraffin. Tablets, coated tablets, capsules, pills and granules can contain the active compound or compounds in addition to the customary excipients, such as (a) fillers and extenders, for example starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, for example carboxymethylcellulose, alginates, gelatine and polyvinylpyrrolidone, (c) humectants, for example glycerol, (d) disintegrating agents, for example agar-agar, calcium carbonate and sodium carbonate, (e) solution retarders, for example paraffin, and (f) absorption accelerators, for example quaternary ammonium compounds, (g) wetting agents, for example cetyl alcohol and glycerol monostearate, (h) adsorbents, for example kaolin and bentonite, and (i) lubricants, for example talc, calcium stearate, magnesium stearate, and solid polyethylene glycols, or mixtures of the substances listed under (a) to (i). The tablets, coated tablets, capsules, pills and granules can be provided with the customary coatings and shells, optionally containing opacifying agents, and can also be of a composition such that they release

the active compound or compounds only or preferentially in a certain part of the intestinal tract, if appropriate in a delayed manner, examples of embedding compositions which can be used would be polymeric substances and waxes. If appropriate, the active compound or compounds can also be presented in microencapsulated form with one or more of the abovementioned excipients. Formulations for parenteral administration are stirilized aqueous solutions, water-insoluble excipients, suspensions, emulsions, and suppositories. Suppositories can contain, in addition to the active compound or compounds, the customary water-soluble or water-insoluble excipients, for example polyethylene glycols, fats, for example cacao fat, and higher esters (for example C14-alcohol with C16-fatty acid) or mixtures of these substances. Ointments, pastes, creams and gels can contain, in addition to the active compound or compounds, the customary excipients, for example animal and vegetable fats, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures of these substances. Dusting powders and sprays can contain, in addition to the active compound or compounds, the customary excipients, for example lactose, talc, silicic acid, aluminum hydroxide, calcium silicate and polyamide powder, or mixtures of

these substances. Sprays can additionally contain the customary propellants, for example chlorofluorohydrocarbons. Solutions and emulsions can contain, in addition to the active compound or compounds, the customary excipients, such as solvents, solubilizing agents and emulsifiers, for example water, ethyl alcohol, isopropyl alcohol, ethylcarbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, in particular cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, glycerol formal, tetrahydrofurfuyl alcohol, polyethylene glycols and fatty acid esters of sorbitan, or mixtures of these substances. For parenteral administration, the solutions and emulsions are also be in a sterile form which is isotonic with blood. Suspensions can contain, in addition to the active compound or compounds, the customary excipients, such as liquid diluents, for example water, ethyl alcohol and propylene glycol, and suspending agents, for example ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances. The formulation forms mentioned can also contain coloring agents, preservatives and additives that improve the smell and taste, for example

peppermint oil and eucalyptus oil, and sweeteners, for example saccharin. The abovementioned pharmaceutical formulations can also contain other pharmaceutical active compounds in addition to the compounds according to the present invention. The abovementioned pharmaceutical formulations are prepared in the customary manner by known methods, for example by mixing the active compound or compounds with the excipient or excipients.

The therapeutically active compounds should preferably be present in the abovementioned pharmaceutical formulations in a concentration of about 0.1 to 99.5, preferably about 0.5 to 95% by weight of the total mixture.

The formulations mentioned can be used on humans and animals orally, rectally, parenterally (intravenously, intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally or locally (dusting powder, ointment, drops) and for the therapy of infections in hollow spaces and body cavities. Possible suitable formulations are injection solutions, solutions and suspensions for oral therapy and gels, infusiton formulations, emulsions, ointments or drops, ophthalmological and dermatological formulations, silver salts and other salts, eardrops, eye onintments, dusting powders or solutions can be used for local therapy. In the case of animals, intake

can also be in suitable formulations via the feed or drinking water. Gels, powders, dusting powders, tablets, delayed release tablets, premixes, concentrates, granules, pellets, boli, capsules, aerosols, sprays and inhalants can furthermore be used on humans and animals. The compounds according to the present invention can moreover be incorporated into other carrier materials, such as for example, plastics (chain of plastic for local therapy), collagen or bone cement.

In general, it has proved advantageous both in human and in veterinary medicine to administer the active compound or compounds according to the present invention in total amounts of about 0.1 to about 15 mg/kg, preferably 0.5 to 2 mg/kg of body weight, 1-3 times every 24 hours, if appropriate in the form of several individual doses, to achieve the desired results. However, it may be necessary to deviate from the dosages mentioned, and in particular to do so as a function of the nature and body weight of the object to be treated, the nature and severity of the disease, the nature of the formulation and of the administration of the medicament and the period or interval within which administration takes place. Thus in some cases it can suffice to manage with less than the abovementioned amount of active compound, while in other cases the abovementioned amount of active compound must be

exceeded. The particular optimum dosage and mode of administration required for the active compounds can be determined by any expert on the basis of his expert knowledge.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

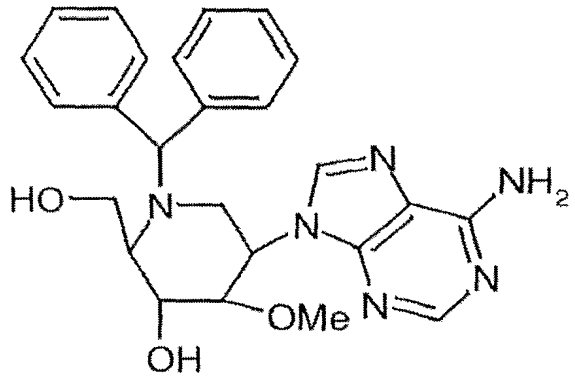
Example 1: Synthesis of oligonucleotide of the SEQ. ID. NO: 1

All of the oligonucleotides were synthesized trityl-on with 1 μM scale using ABI 392 DNA/RNA synthesizer (Applied Biosystem). The method is described in Korea Patent Application No. 99-26947. Time for general condensation is 1 min, whereas was 10 min in case of nucleotides of the present invention containing azasugar with other functional groups than hydrogen at position 4. Solid support and protecting

group were removed by heating with ammonium hydroxide for 17 hours at 55℃, and this solution was freeze-dried by adding 5 drops of trimethylamine every hour to keep the protecting group, dimethoxytrityl (DMT). The residue was dissolved in 1 ㎖ of 100 mM triethylammonium acetate(TEAA, pH 7.0), and then purified by reversed phase high-performance liquid chromatography(RP-HPLC, Hamilton PRP-1, 300 ㎜ x 7 ㎜, 18-28% acetonitrile/100 mM TEAA, pH 7, monitored at 260 nm). The desired fractions were freeze-dried and the residual TEAA was removed by freeze-drying twice after adding 1 ㎖ of distilled water thereto. The residual solid was re-dissolved thoroughly by adding 0.3 ㎖ of 80% acetic acid and vortexing, and dimethoxytrityl group was removed by incubating for 20 minutes at room temperature. Ethanol (0.3 ㎖) was added to the above solution to remove excess acetic acid, and then freeze-dried. The sample was re-dissolved by vortexing in 1 ㎖ of distilled water, and then 1 ㎖ of ether was added thereto and vortexed again. After ether layer was removed by pipette, 1 ㎖ of ether was added thereto again. This procedure was repeated twice. Water layer was collected for freeze-drying. Dried residue was dissolved in 1 ㎖ of distilled water, and then quantified by UV absorbance at 260nm at 70℃. The extinction coefficients of natural nucleotides (at 260nm) used for calculation were as follows: dAMP,

15200; dCMP, 7700; TMP, 8830; dGMP, 11500. The extinction coefficients of nucleotides having azasugar were considered to be same as those of natural nucleotides. The base composition of the purified oligonucleotides was confirmed by total digestion with nuclease followed by RP-HPLC (Hewlett Packard, ODS hypersil, C-18; 20 mM K_2HPO_4, pH 5.6(A), MeOH (B), 100% A to 40% B in 20 min) and laser desorption mass spectrometry. Finally, oligonucleotide of the SEQ. ID. NO: 1 containing adenosine derivative(ⓐ) having the structure of <Chemical Formula 3> at various position in its base sequence was synthesized.

<Chemical Formula 3>



Example 2 ~ Example 29: Synthesis of oligonucleotides of the SEQ. ID. NO: 2 ~ 29

After synthesis of the adenosine derivative having the structure of <Chemical Formula 3> using the method described in <example 1>, oligonucleotides of the SEQ. ID. NO: 2 ~ 29 containing the adenosine derivative at various position in its nucleotide sequence were synthesized.

Oligonucleotides were synthesized, whose sequences were complementary to TAR sequences of HIV-1 and SIV (simian immunodeficiency virus), IRES (internal ribosomal entry site) of poliovirus, and sd (splicing donor), LTR and gag of HIV. In addition, the oligonucleotides of the present invention include random oligonucleotides (represented by the SEQ. ID. NO: 20 ~ 23) having random base sequence without target gene. The oligonucleotide of the SEQ. ID. NO: 29 was designed to have CpG sequence that had been reported to induce strong immune response in vivo (A. M. Crieg, et al., *Nature*, 271, 546, 1995; D. E. McFarlane and L. Manzel, *J. Immunol.*, 160, 1122, 1998; A. Yi, et al., *J. Immunol.*, 160, 4755, 1998). Most of the oligonucleotides of the present invention have P=S backbone in which oxygen of P=O bond is substituted with sulfur. But oligonucleotides of the SEQ. ID. NO:

5, 13 and 24 have P=O backbone.

Base sequences, positions of adenosine derivative ⓐ and target genes of the synthetic oligonucleotides were summarized in Table 1.

<Table 1>

Oligonucleotide list

Example NO:	Sequence	Back bone	Target site	SEQ.ID. NO:
Example 1	AGC TCC CⓐG GCT CⓐG ATC	P=S	HIV-1 TAR	1
Example 2	ⓐGC TCC CⓐG GCT CⓐG ATC	P=S	HIV-1 TAR	2
Example 3	ⓐGC TCC CⓐG GCT CAG ⓐTC	P=S	HIV-1 TAR	3
Example 4	AGC TCC CAG GCT CAG ATC	P=S	HIV-1 TAR	4
Example 5	ⓐGC TCC CⓐG GCT CAG ⓐTC	P=O	HIV-1 TAR	5
Example 6	ⓐTC TGC TCⓐ GAG ATA CⓐA	P=S	SIV TAR	6
Example 7	ⓐGT CⓐC TCA GGⓐ CTC TGG	P=S	SIV TAR	7
Example 8	ⓐCT CAT CⓐG CCT ⓐAG CTA	P=S	Polio IRES	8
Example 9	ⓐGC TCC CAG GCT CAG ⓐTC	P=S	HIV-1 TAR	9
Example 10	ⓐTT TTT GGC CTⓐ CTC ⓐCC	P=S	HIV-1 sd	10
Example 11	ⓐTT GⓐG GCT TⓐA GCⓐ GTG	P=S	HIV-1 LTR	11
Example 12	CⓐG AAT ⓐGA GGⓐ CTG CTⓐ T	P=S	HIV-1 gag	12
Example 13	AGC TCC CAG GCT CAG ATC	P=O	HIV-1 TAR	13
Example 14	ⓐGC TCC CⓐG GCT CAG ATC	P=S	HIV-1 TAR	14
Example 15	ⓐGC TCC CAG GCT CⓐG	P=S	HIV-1 TAR	15

Example 16	ⓐGC TCC CⓐG	P=S	HIV-1 TAR	16
Example 17	ⓐGC TCC CⓐG GCT CⓐG ⓐTC	P=S	HIV-1 TAR	17
Example 18	ⓐ GⓐG ⓐGC TCC CⓐG GCT CⓐG ⓐT	P=S	HIV-1 TAR (475-495)	18
Example 19	ⓐGⓐ GCT CCC ⓐGG CTC ⓐGⓐT	P=S	HIV-1 TAR	19
Example 20	ⓐGC TCC ⓐGC TCC ⓐGC TCC ⓐG	P=S	Random 1	20
Example 21	ⓐGC TCⓐ GCT CⓐG CTC ⓐGC TⓐG	P=S	Random 2	21
Example 22	ⓐCT CⓐC TCⓐ CTC ⓐCT CⓐC	P=S	Random 3	22
Example 23	ⓐCG ⓐCG ⓐCG ⓐCG ⓐCG ⓐCG ⓐC	P=S	Random 4	23
Example 24	DMT-ⓐGGGⓐG	P=O	Random 5	24
Example 25	ⓐTGGGGⓐT	P=S	G-quartet	25
Example 26	$A^0G^0C^0$ $T^*C^*C^*$ $C^*ⓐ^*G^*$ $G^*C^*T^*$ $C^*ⓐ^*G^0$ A^0T^0C	^{0}P=O, *P=S	HIV-1 TAR	26
Example 27	$A^*G^0C^*$ $T^0C^*C^0$ $C^*ⓐ^0G^*$ $G^0C^*T^0$ $C^*ⓐ^0G^*$ A^0T^*C	^{0}P=O, *P=S	HIV-1 TAR	27
Example 28	$A^0G^*C^*$ $T^*C^*C^*$ $C^*ⓐ^*G^*$ $G^*C^*T^*$ $C^*ⓐ^*G^*$ A^*T^0C	^{0}P=O, *P=S	HIV-1 TAR	28
Example 29	ⓐCG CⓐC GCⓐ CTC ⓐCG CⓐC	P=S	CpG-Random	29
Example 30	ⓐ monomer			30

P=S: phosphorothioate backbone,

P=O: phosphodiester backbone,

^{0}P=O, *P=S: chimera (mixed forms containing phosphorothioate and phosphodiester backbones).

Experimental example 1: Anti-viral activity of oligonucleotides against HIV-1 proliferation

In order to measure the anti-viral activity of oligonucleotides of the present invention against HIV replication, Jurkat-Tat cells were infected with HXBc2/ Δ tat virus (tat-defective HIV-1, NIH AIDS Research and Reference Reagent program, USA). Antiviral activity of each oligonucleotide was measured by analysis of syncytium formation or reverse transcriptase (RT) activity in the presence or absence of each oligonucleotide in the cultures of the HIV-1 infected cells.

<1-1> Syncytium formation experiment

HXBc2/Δ tat virus (laboratory strain of tat-defective HIV-1) and Jurkat-Tat cells (Tat-expressing Jurkat cells) were obtained from Dr. Sodroski at Dana-Farber Cancer Institute, Harvard Medical School, and used in the experiments of the present invention. When infected with HXBc2/ Δ tat, Jurkat-Tat cells express HIV-1 envelop glycoproteins (gp120 and gp41) on the cell surface, which causes cell fusion by interaction between gp120 and CD4 molecules on the infected and uninfected cells, respectively, resulting in production of syncytia, multinucleated giant cells. Based on this

principle, anti-HIV-1 activity of each oligonucleotide of the present invention was examined by the relative amounts of syncytia formed in the culture of infected cells in the presence of each oligonucleotide. Jurkat-Tat cells were washed twice with PBS, and then infected with HXBc2/Δ tat virus ($TCID_{50}$ 50/1x10^5 cells) in a serum-free medium for 1 hour at 37℃, CO_2 incubator. After infection, cells were washed with PBS buffer to remove uninfected virus, and then cultured in the 24-well plate (2x10^5 cells/well). Medium supplemented with 10% FBS ($RPMI_{10}$) or serum-free medium (Opti-MEM) were used for culturing. In each case, oligonucleotides(0.1 μM) of the present invention synthesized in example 1 were added at the beginning of culture. In order to measure the long-lasting anti-viral activity of the oligonucleotides of the present invention, the infected cells were cultured in the presence of each oligonucleotide and the dates of syncytium formation and the number of syncytium were observed for 12 days after a single treatment. These results were summarized in Table 2 and Table 3. AZT was also tested for its anti-HIV-1 activity at the same concentration (0.1 μM) as a standard comparative anti-HIV-1 agent.

<Table 2>

Anti-HIV-1 activity of oligonucleotides in serum-free

medium (Opti-MEM)

Days after Infection	1~3	4	5	6	7	8	9	10	11	12
Control			+	++	+++	+++	++++	++++	+++++	+++++
Positive Control (AZT)			-	-	+/-	+	++	++	+++	+++
SEQ. ID. NO: 1			-	-	+/-	+/-	+	++	++	++
SEQ. ID. NO: 2			-	-	-	+/-	+/-	++	++	++
SEQ. ID. NO: 3			-	+/-	+/-	+/-	+/-	+	+++	+++
SEQ. ID. NO: 4		+	++ +	++++	++++	++++	++++	++++	+++++	+++++
SEQ. ID. NO: 5		+	++	+++	+++	+++	+++	++++	+++++	+++++
SEQ. ID. NO: 8			-	+/-	+/-	++	++	+++	+++	+++
SEQ. ID. NO: 9			-	+/-	+	+	++	+++	+++	+++
SEQ. ID. NO: 10			-	-	-	+/-	++	++	+++	+++
SEQ. ID. NO: 11			-	+/-	+/-	+/-	+	++	+++	+++
SEQ. ID. NO: 12			-	+/-	+/-	+/-	+/-	+	++	+++
SEQ. ID. NO: 13			+	++	++	+++	++++	++++	+++++	+++++
SEQ. ID. NO: 14			-	-	+/-	+/-	++	+++	+++	++++
SEQ. ID. NO: 15			-	+/-	+/-	+/-	+/-	+	++	+++
SEQ. ID. NO: 16			-	+/-	+/-	+/-	+/-	+	++	+++
SEQ. ID. NO: 17			-	-	-	+/-	+/-	+/-	+/-	+/-
SEQ. ID. NO: 18			-	-	-	+/-	+/-	+/-	+	+
SEQ. ID. NO: 19			-	-	+/-	+/-	+	+	+	++
SEQ. ID. NO: 20			-	-	-	+/-	+/-	+/-	+/-	+
SEQ. ID. NO: 21			-	-	+/-	+/-	+/-	+	+	+
SEQ. ID. NO: 22			-	-	-	+/-	+/-	+/-	+/-	+/-
SEQ. ID. NO: 23			-	-	+/-	+/-	+/-	+	+	+

SEQ. ID. NO: 24			-	+/-	+	+	++	+++	+++	+++
SEQ. ID. NO: 25			-	+/-	+/-	+	++	+++	+++	+++
SEQ. ID. NO: 26			-	+/-	+/-	+/-	+/-	++	++	+++
SEQ. ID. NO: 27			-	+/-	+	++	+++	+++	+++	++++
SEQ. ID. NO: 28			-	+/-	+/-	+	++	++	++	+++
SEQ. ID. NO: 29			-	-	+/-	+/-	+/-	+/-	+	+

*Syncytium Number: +/-:<10, +:~20, ++:~50, +++:~100, ++++:~200, +++++:>500

<Table 3>

Anti-HIV-1 activity of oligonucleotides in medium supplemented with 10% PBS ($RPMI_{10}$)

Days after Infection	0~1	2	3	4	5	6	7	8	9
Control	-	+	+	++	++	++++	++++	+++++	+++++
Positive Control (AZT)	-	-	-	+/-	+/-	+	++	+++	++++
SEQ. ID. NO: 2	-	-	-	-	+/-	+/-	+/-	+	++
SEQ. ID. NO: 4	-	++	++++	++++	++++	++++	++++	+++++	+++++
SEQ. ID. NO: 11	-	-	-	-	-	+/-	+/-	++	++
SEQ. ID. NO: 17	-	-	-	-	-	-	+/-	+	+
SEQ. ID. NO: 22	-	-	-	-	-	-	-	+/-	+/-
SEQ. ID. NO: 29	-	-	-	-	-	-	-	+/-	+

* Syncytium Number: +/-:<10, +:~20, ++:~50, +++:~100, ++++:~200, +++++:>500

As shown in Table 2, oligonucleotides of the SEQ.

ID. NO: 2, 3, 11, 17-23 and 29 showed higher anti-viral activity than that of AZT. Especially, oligonucleotides of the SEQ. ID. NO: 17, 20 and 22 showed remarkable anti-viral activity. The oligonucleotide showing anti-HIV-1 activity comprises P=S backbone and more than 3 six-membered azasugar nucleotides in the sequence. Among them, oligonucleotides of the SEQ. ID. NO: 20-23 and 29 showed strong anti-HIV-1 activity though they had no sequence specificity to HIV-1 gene. Oligonucleotide of the SEQ. ID. NO: 4 comprising P=S backbone and no six-membered azasugar nucleotide showed enhanced severity in syncytium formation rather than inhibition (Fig. 1).

Besides, the oligonucleotide of the SEQ. ID. NO: 5 comprising P=O backbone and 3 six-membered azasugar nucleotides, also caused a syncytium formation earlier than untreated control when treated in the culture of HIV-1 infected cells, but the number of syncytium was not higher than that of control. Both of the oligonucleotides of the SEQ. ID. NO: 4 and 5 seemed to accelerate HIV proliferation in the infected cells at the concentration of 0.1 μM. Oligonucletide of the SEQ. ID. NO: 13 comprising P=O bond but no six-membered azasugar or oligonucleotides of the SEQ. ID. NO: 26 to 28 comprising partial P=O/P=S backbone (regardless of the presence of six-membered azarsugar nucleotides) did not show any discernable anti-HIV-1 activity. While no

clear rule was provided by this invention, anti-viral activity of the oligonucleotides of the present invention seemed to depend on the position or number of azasugar nucleotides in the sequence, and the oligonucleotides containing more than 4 azasugar nucleotides seemed to be much more efficient to inhibit HIV-1 replication.

When treated in a serum-free medium (Opti-MEM), the oligonucleotides of the SEQ. ID. NOs: 2, 11, 17, 22 and 29 inhibited HIV-1 proliferation efficiently even at the final concentration of 0.06 μ M. While, the oligonucleotide of the SEQ. ID. NO: 4 showed no anti-viral activity in the same condition of serum-free medium even at the concentration of 0.1 μ M (Fig. 2).

As shown in Table 2 and Table 3, when HIV-1-infected cells were cultured in the serum-containing medium, syncytium formation was observed 2-3 days earlier, and the number of syncytia was higher than those shown in serum-free medium. It means that HIV-1 proliferation was accelerated when serum was added.

While anti-viral activity of oligonucleotides of the SEQ. ID. NOs: 2, 11 and 17 was markedly reduced in the serum-containing medium, the oligonucleotides of the SEQ. ID. NOs: 22 and 29 was merely or not affected for their anti-viral activity by the existence of serum. The 5 oligonucleotides mentioned above were much more

efficient than AZT for a long-lasting anti-HIV-1 activity even in serum-existing condition. Anti-viral activity of each oligonucleotide was measured at varying concentrations in serum-containing medium and summarized in Table 4.

<Table 4>

Anti-HIV-1 activity of oligonucleotides treated with different concentrations in serum-existing condition.

SEQ.ID. NO: / Conc. (μM)	Days after Infection*										
	2	4	11	17	18	19	20	21	22	23	28
0	3	3	3	3	3	3	3	3	3	3	3
0.015	5	2~3	5	3	5	3	5	3	6	4	6
0.03	5	2	5	6	4	4~5	5	3	6~7	5	6~7
0.06	5	2~3	6	6~7	5	5	6~7	6~7	6~7	5	7
0.125	6	2~3	6	8	9	6~7	8	8~9	9	7	9
0.25	8~9	2	8	8~9	-	11	-	11	-	8~9	11
0.5	9	2~3	9~10	11	-	-	-	-	-	-	-
1	-	3	-	-	-	-	-	-	-	-	-

*: Dates when the first syncytium was observed after infection. HXBc2/Δ tat-infected Jurkat-Tat cells were cultured in the presence of oligonucleotides at varying concentrations in a medium supplemented with FBS (RPMI1640-10% FBS).

While first syncytium was observed at day 3 after infection in untreated control, the culture treated

with the oligonucleotide of the SEQ. ID. NO: 22 did not show any syncytium formation even at the concentration of 0.015 μM until 6 days had passed since infection. HIV proliferation was markedly inhibited by the oligonucleotide 22 even at a low concentration, and no syncytium was observed at 0.25 μM or over. Together with the oligonucleotide of the SEQ. ID. NO: 22, oligonucleotides of the SEQ. ID. NOs: 20, 21 and 29 comprising random sequence and oligonucleotides of the SEQ. ID. NOs: 11 and 17 complementary to HIV-1 TAR sequence effectively inhibited HIV-1 proliferation at 0.06 μM. It required 0.5 μM of these oligonucleotides to make sterile inhibition of HIV-1 proliferation. Even though the oligonucleotide of the SEQ. ID. NO: 18 showed higher EC_{50} value(0.12 μM) than that of oligonucleotide of the SEQ. ID. NO: 17, lower amounts (0.25 μM) of oligonucleotide 18 were required for sterile inhibition of HIV-1 proliferation than that of 17. It means that the oligonucleotide of the SEQ. ID. NO: 18 was more effective than oligonucleotide of the SEQ. ID. NO: 17 for the inhibition of virus proliferation. When treated in a serum-free medium (Opti-MEM), oligonucleotide of the SEQ. ID. NO: 2 was similar to that of the oligonucleotide of the SEQ. ID. NO: 22 for their anti-viral activity at 0.1 μM. On the other hand, in the presence of serum in medium, anti-viral activity of the oligonucleotide of the SEQ.

ID. NO: 2 was significantly attenuated, while that of oligonucleotide 22 was not.

These results suggested that the serum effects on the anti-HIV-1 activity of oligonucleotides seem to depend on the oligonucleotide sequence. To see the serum effects more precisely, each oligonucleotide was tested for its anti-HIV-1 activity in the presence of different kinds and varying concentrations of sera, and then summarized in Table 5.

<Table 5>

Serum effects on the anti-HIV-1 activity of each oligonucleotide with different kinds and concentrations of sera.

SEQ.ID.NO: Serum Types & Conc.**		Days after Infection*						
		Control	2	4	11	20	22	29
FBS	0 %	4	8	4	8	8	8	8
	5 %	3	5	2	6	7	8	8
	10 %	3	5	2	6	7	8	7~8
	50 %	3	5	2	5~6	6~7	7~8	7~8
Human serum 10%		3	5	2	6	7	8	8
Horse serum 10%		3	5	2	6	7	8	8

* Dates when the first syncytium was observed after HIV-infection. Infected cells were cultured in the presence of each oligonucleotide, and different types

and varying concentrations of sera.

** Opti-MEM was used for a serum-free medium and RPMI was used for a serum-supplemented medium.

*** Oligonucleotide concentration: 0.1 μ M.

As shown in Table 5, not the serum types and concentrations but the existence of serum affected anti-viral activity of the oligonucleotides. Among those, the oligonucleotides of the SEQ. ID. NO: 22 and 29 were merely affected for its anti-HIV-1 activity by the existence of serum, while anti-viral activity of the oligonucleotide of the SEQ. ID. NO: 2 was markedly reduced when serum was added in comparison with those of other oligonucleotides. It means that the oligonucleotides of the present invention, such as SEQ. ID. NO: 20, 22, and 29, can be used as an AIDS therapeutic drug in vivo because of its resistance to the serum effects.

<1-2> Reverse transcriptase assay

Jurkat-Tat cells were infected with HXBc2/Δ tat virus as described in the experimental example <1-1>, and then 2/3 volume of culture medium containing cells was harvested at an interval of 3 days. Fresh medium and the test compounds were replaced for further culturing. The harvested culture medium was vortexed vigorously for 1 minute, followed by centrifugation for

10 seconds at 15,000 rpm to remove cell debris.

Reverse transcriptase activity was measured by the method reported previously with a slight modification. Specifically, the supernatant was transferred into a new test tube, to which PEG/NaCl solution equivalent to 1/2 volume of supernatant (30% PEG in 0.4 M NaCl) was added. Mixed thoroughly, the sample was left overnight at 4℃. The solution was centrifuged for 45 minutes at 15,000 rpm. After removal of supernatant, 10 ㎕ of lysis buffer(0.25% Triton X-100, 20% glycerol, 50 mM Tris-HCl pH 7.5, 0.1% DTT, 250 mM KCl) was added to the virus sediments for dissolution. Three cycles of freezing/thawing were repeated by putting in Dry-ice/ethanol and in 37℃ water-bath to crush the above sediments. Reverse transcriptase reaction solution(1 unit/㎕ of RNasin, 1 μ Ci of ^{3}H-TTP, 0.025 unit Poly(A)-(dT), 50 μ M Tris-HCl pH 7.5, 5 mM DTT, 0.1% Triton X-100) was added into each test tube(50 ㎕/test tube), and incubated for 1 hour at 37℃. After reaction, the solution was absorbed into DE51 filter paper (Whatman). The filter paper was dried, washed with 2X SSC solution (3 M NaCl, 0.3 M sodium citrate, pH 7.0) 3 times and 95% ethanol twice, and then dried. The filter paper was transferred into a scintillation vial, and 3-5 ㎖ of scintillation cocktail was added thereto. Finally, the amount of incorporated radioactivity was measured by liquid scintillation counter, and the results were

shown in Fig. 3 and Fig. 4.

As shown in Fig. 3 and Fig. 4, in the serum-free medium (Opti-MEM), the reverse transcriptase activity was rapidly increased from day 6 till day 12 after infection. After day 12, the enzyme activity was decreased and maintained at a certain level. Meanwhile, when the cells were cultured in medium containing 0.5 μ M oligonucleotides of the SEQ. ID. NO: 2 and 22 of the present invention, the reverse transcriptase activity was not increased at all until 18 days after infection (Fig. 3).

These results demonstrate that the anti-HIV activity of the oligonucleotides of the present invention was not affected much by the presence of serum when used at such a high concentration as 0.5 μ M.

Anti-HIV-1 activities of oligonucleotides of the SEQ. ID. NO: 2, 17, 20 and 22 in the serum-supplemented medium were similar to those shown in serum-free medium when measured by reverse transcriptase activity (Fig. 4). However, for the oligonucleotide of the SEQ. ID. NO: 4 comprising P=S backbone without six-membered azarsugar nucleotides, the reverse transcriptase activity was not inhibited, rather increased at earlier days after infection than that of control, which indicated that viral replication was accelerated by the

treatment of oligonucleotide of the SEQ. ID. NO: 4 as shown in serum-free medium.

<Experimental Example 2> Anti-viral activity of oligonucleotides against various HIV-1 variants

The present inventors have further investigated whether the oligonucleotides of the present invention showed anti-viral activity against IIIB and $SHIV_{89.6}$ (obtained from AIDS Research and Reference Reagent Program, NIH, USA) in addition to HXBc2/Δ tat(tat-defective HIV-1 strain).

C8166 cells (obtained from AIDS Research and Reference Reagent Program, NIH, USA) were infected with $SHIV_{89.6}$ as described above experimental example <1-1>. The number of syncytium formed in the presence of oligonucleotides of the present invention (0.2 μ M and 0.5 μ M) was determined at the following days after infection and summarized in Table 6.

<Table 6>

Anti-viral activity of oligonucleotides against $SHIV_{89.6}$

Sample	Days after Infection Conc.	1	2	3	4	5	6	7
Control	0 μM	-	+/-	+	++	++++	+++++	+++++
SEQ.ID. NO: 2	0.2 μM	-	-	-	+/-	+	++	++++
	0.5 μM	-	-	-	-	-	-	-

SEQ.ID. NO: 4	0.2 μM	-	+/-	++	++++	+++++	+++++	+++++
	0.5 μM	-	+/-	++	++++	+++++	+++++	+++++
SEQ.ID. NO: 17	0.2 μM	-	-	-	+/-	+	+++	++++
	0.5 μM	-	-	-	-	-	-	-
SEQ.ID. NO: 20	0.2 μM	-	-	-	-	-	-	-
	0.5 μM	-	-	-	-	-	-	-
SEQ.ID. NO: 22	0.2 μM	-	-	-	-	-	-	-
	0.5 μM	-	-	-	-	-	-	-

*Syncytium number; -: no, +/-: <10, +: >20, ++: >40, +++: >60, ++++: >80, +++++: >100

As shown in Table 6, syncytium formation was not observed when infected cells were treated with the oligonucleotides of the SEQ. ID. NO: 20 and 22 even at the final concentration of 0.2 μ M. While the oligonucleotides of the SEQ. ID. NO: 2 and 17, showed sterile inhibition of syncytium formation during the experiment when the infected cells were treated at 0.5 μ M, they were not so effective when treated at 0.2 μ M, and the number of syncytium increased as time went on after first detection on 4 days after infection. On the other hand, syncytium formation began 2 days after infection in untreated control or in culture treated with the oligonucleotide of the SEQ. ID. NO: 4. Since then, the number of syncytium was remarkably increased. Particularly, in case of the oligonucleotide of the SEQ. ID. NO: 4, syncytium formation was observed earlier than that shown in untreated control (Table 6).

Anti-viral activity of each oligonucleotide of the present invention against various HIV-1 varients was determined by the tetrazolium-based colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. MT-4, Jurkat-Tat and C8166 cells were infected with HIV-1 IIIB, HXBc2/Δ tat and $SHIV_{89.6}$, respectively, as described in the Experimental example 1. Four days after infection, the antiviral activity of each oligonucleotide was monitored by MTT assay, and then expressed by EC_{50}, concentration required to inhibit the 50% of the viral infection, summarized in Table 7.

<Table 7>

Anti-viral activity of oligonucleotides against various HIV-1 variants

Samples (SEQ.ID.NO:) Virus/Host	EC_{50} (μM)								
	2	4	17	20	22	29	AZT	ddC	ddI
HXBc2/Δtat /Jurkat-Tat	0.12	>10	0.06	0.08	0.05	0.08	0.01	ND*	ND
HIV-1 IIIB /MT-4	0.13	>2.0	0.14	0.10	0.08	0.12	0.007	0.659	35.9
$SHIV_{89.6}P$ /C8166	0.23	>2.0	0.16	0.12	0.10	0.16	>400	2.673	70.7

*ND: Not performed.

As a result, the oligonucleotides of the SEQ. ID. NO: 2, 17, 20, 22 and 29 showed effective anti-viral

activity against virulent strains such as HIV-1 IIIB and HIV-$1_{89.6P}$(0.05 μ M < EC_{50} < 0.12 μ M)as well as against laboratory strain such as HXBc2/Δtat (0.05 μ M < EC_{50} < 0.12 μ M). Among them, the oligonucleotide of the SEQ. ID. NO: 22 was the most potent for its antiviral activity against a broad spectrum of HIV-1. It is particularly noted that the anti-viral activity of the oligonucleotides of the present invention (NO: 2, 17, 20, 22 and 29) was much more effective than those of other AIDS therapeutic drugs such as ddC or ddI.

The anti-viral activity of AZT (Sigma, USA), widely used for AIDS therapeutic drug, against HXBc2/ Δ tat and IIIB was about 10-times as strong as those of oligonucleotides of the present invention for a short period of time (for 4 days) after infection, but the oligonucleotides of the present invention were much more effective than that of AZT for a long term anti-viral activity (for 9-12 days) after a single treatment(Table 2 and Table 3). Meanwhile, AZT did not show anti-viral activity at all against SHIV-$1_{89.6P}$.

These results suggest that the oligonucleotides of the present invention maintain their activity longer than AZT because of their stability in the medium. Also it could be expected that the oligonucleotides of the present invention are to be effective for the

treatment of the AIDS patients who have drug-resistant mutant against the commercialized RT (reverse transcriptase) inhibitors such as AZT, ddC, ddI etc, since the antiviral mechanism of the oligonucleotides of the present invention is quite different from that of RT inhibitors.

<Experimental Example 3> Cytotoxicity test of oligonucleotides

Following experiment was performed to confirm the cytotoxicity of oligonucleotides of the present invention.

In order to check the cytotoxicity of oligonucleotides, C8166, 174xCEM and MT-4 cells (AIDS Research and Reference Reagent Program, NIH, USA), HeLa (ATCC CCL 2), Jurkat E6 (ATCC TIB 152), Vero (ATCC CCL 81) and U-937 (ATCC CRL 1593) cells were used. Each cell lines were treated with oligonucleotides for 4 days at varying concentrations, and then the viable cells were measured using modified MTT assay. Specifically, MT-4 cells ($1x10^5$ cells/well) in RPMI1640 supplemented with 10% FBS was loaded into 96-well microtiter plate (200 ㎕/well), and different concentrations (0 μM - 100 μM) of oligonucleotides were added thereto, followed by culturing for 4 days. 10 ㎕ of MTT solution (7.5 ㎎/㎖, Sigma Chem. Co., dissolved in PBS, stored at 4℃) was added to each well

and the cultures were incubated for 4 hours at 37℃. Removing supernatant carefully, 100 ㎕ of HCl solution containing isopropanol(0.4 M) was added. After leaving it for 5 minutes at room temperature, absorbance was measured at 570nm with ELISA reader to quantify the viable cells. The cytotoxic concentration 50 (CC_{50}) was determined by the concentration of the compound required for 50% reduction of the cell viability. When the CC_{50} was above 100 μ M, cytotoxicity of the sample was not re-determined at the concentration more than this. Cytotoxicity of each oligonucleotide of the present invention was summarized in Table 8.

<Table 8>

Cytotoxicity of oligonucleotides

Cell line / SEQ.ID.NO:	CC_{50}(μ M)						
	MT-4	Jurkat	C8166	Vero	CEMx174	U937	HeLa
2(ⓐ:3)	>100	80	80	>100	>100	40	>100
4(ⓐ:0)	>100	80	90	60	80	20	>100
11(ⓐ:4)	>100	>100	60	>100	>100	40	>100
13(P=O)	80	>100	>100	>100	>100	>100	>100
17(ⓐ:4)	>100	>100	55	>100	>100	45	>100
20(ⓐ:4)	>100	>100	>100	>100	>100	40	>100
22(ⓐ:5)	>100	>100	>100	80	>100	50	>100
29(ⓐ:5)	>100	80	>100	80	>100	50	>100

*The number in parentheses is the number of six-membered azasugars contained in each oligonucleotide.

*CC_{50}: 50% cytotoxic concentration of the

oligonucleotide.

*Cytotoxicity was expressed by CC50 value derived from the results of MTT assay on day 4 after treating cells with oligonucleotides at varying concentrations.

As shown in Table 8, CC_{50} values of oligonucleotides of the present invention were above 50 μM in most cells except U937. It means that the TI (Therapeutic Index represented by CC_{50}/EC_{50}, N. Jing, et al., *J. Biol. Chem.*, 275, 3421-3430, 2000) was over 100, especially, TI of the oligonucleotides of the SEQ. ID. NO: 22 and 29 was over 250 even in U937 cells. The TI value in medication represents its safety and effectiveness. The greater TI is the safer and more effective the drug is. Considering all these together, it was expected that the oligonucleotides of the present invention might not cause any harmful effect when used as a therapeutic drug in vivo.

<Experimental example 4> Oligonucleotide stability test

To test stability of oligonucleotides of the present invention, oligonuclotides were added (final conc. 13 μM) into culture supernatant (Fig. 5) or fresh medium (RPMI1640 supplemented with 10% FBS) (Fig. 6), and incubated at 37℃, CO_2 incubator. From each reaction, 10 μl was withdrawn at 0, 1, 2 hours and 1st, 2nd, 4th, 6th day for culture supernatant, and at 0, 1st,

2^{nd}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} day for fresh medium, respectably, and these aliquots were run on 20% SDS-PAGE electrophoresis. The stability of each oligonucleotide of the present invention was determined by the band integrity after staining the gel with ethidium bromide solution.

As shown in Fig. 5, oligonucleotide having P=O backbone without six-membered azasugar-containing nucleotides (represented by the SEQ. ID. NO: 13) was completely degradated in culture supernatant within 2 hours by various nuclease secreted by the cells. However, oligonucleotides having P=S backbone (represented by the SEQ. ID. NO: 2 or 4) or oligonucleotide having P=O backbone with six-membered azasugar-containing nucleotides (represented by the SEQ. ID. NO: 5) were not degraded at all for 6 days. Therefore, the oligonucleotides of the present invention are expected to be effective enough to overcome the limitation of the instability of the general antisense oligonucleotides in vivo. In addition, it was noteworthy that even a nucleotide having P=O bonds was resistant against nuclease, if it has more than 3 six-membered azasugar nucleotides, which seemed to be an important information for designing the antisense oligonucleotides.

As shown in Fig. 6, oligonucleotides (the SEQ. ID. NO: 2 and 22) of the present invention keep their stability for over 15 days in fresh medium containing 10% FBS. These results suggest that the oligonucleotides of the present invention can be used as an effective anti-HIV-1 agent without losing anti-viral activity for a long time when they are administered even in vivo.

<Experimental Example 5> Permeability and anti-viral activity of oligonucleotides within cells

<5-1> Membrane permeability of oligonucleotides.

In order to measure the permeability of oligonucleotides into cell membrane, MAGI cells(HeLa cell line containing LTR-β-galactosidase gene, designed to express β-galactosidase under the control of LTR promoter and HIV-1 Tat protein), pSV2-Tat plasmid(Tat-expressing vector) and LTR-CAT plasmid(AIDS Research and Reference Reagent Program, NIH, USA) were used.

To investigate whether the anti-viral activity of oligonucleotides was due to the intracellular sequence-specific antisense mechanisms or not, following experiments were conducted. The oligonucleotides of the present invention were added directly into medium (final conc. 0.25 μM, Fig. 7A) after $1x10^6$ MAGI cells were transfected with 2 μg of pSV2-Tat plasmid using

GenePorter™ kit (Gene Therapy Systems, Inc. San Diego, CA, USA). Otherwise, cells were transfected with 2 ㎍ of pSV2-Tat plasmid together with varying concentrations of oligonucleotides (Fig. 7B). 48 hours after transfection, medium was removed, and cells were fixed for 5 minutes with 1% formaldehyde and 0.2% glutaraldehyde. Fixed cells were washed with PBS and stained with 0.04% X-gal staining solution(4 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.04% 5-bromo-4-chloro-3-indoryl-β -D-galactopyraoside in PBS) for 50 minutes at 37℃. After staining, blue-colored cells were counted to measure the inhibition effect of oligonucleotides against LTR/Tat activity (Fig. 7).

When oligonucleotides of the SEQ. ID. NO: 2, 4 and 22 were added directly into medium, the number of blue-colored cells was similar to that of control, suggesting that the oligonucleotides of the present invention could not suppress LTR activity of the Magi cells probably due to the lack of permeability of the oligonucleotides to the cell membrane (Fig. 7A). Meanwhile, another possibility was raised that this result might be due to the lack of sequence-specific antisense activity to the LTR target site even though they might have normal membrane-penetrating capacity. To avoid this possibility, cells were transfected with pSV2-Tat and oligonucleotides simultaneously, and then blue-colored cells were counted. The number of blue-

colored cells was markedly decreased by transfection of the oligonucleotide of the SEQ. ID. NO: 4, which has no six-membered azasugar nucleotide. On the other hand, transfection of the oligonucleotides of the SEQ. ID. NO: 2 and 22 having 6-membered azasugar nucleotides did not suppress the number of the blue-colored cells (Fig. 7B). These results suggest that the anti-HIV-1 activity of the six-membered azarsugar-containing oligonucleotides of the present invention was unlikely due to the sequence-specific antisense mechanism after membrane-penetration.

To analyze the above result quantitatively, cells were transfected with 2 ㎍ of pSV2-Tat plasmid and various concentrations(0, 0.01, 0.1, 0.5, 1, 2.5 and 5 ㎍) of oligonucleotides simultaneously. Forty-eight hours after transfection, cells were fixed and stained. The number of blue-colored cells due to LTR/Tat-mediated β-galactosidase expression were counted and shown in a percentage(%) to that of control(Fig. 8).

As a result, when the oligonucleotide of the SEQ. ID: 4 was transfected into cells, it suppressed LTR/Tat activity in proportion to the concentration of the oligonucleotide. However, when the oligonucleotides showing anti-viral activity in medium (the SEQ. ID. NO: 2, 17 and 22) were transfected into cells, they did not inhibit LTR/Tat activity. These results indicate that the oligonucleotides of the present invention could not

penetrate cell membrane or their anti-viral activities were not sequence-specific to LTR. Rather, it is expected that the anti-viral activity of the oligonucleotides of the present invention was likely mediated by inhibiting the attachment of HIV-1 to the target cells. In fact, non-specific binding of antisense oligonucleotides to the cell surface proteins has been reported (P. Hawley, et al., *Antisense Nucleic Acid Drug Dev.*, 9, 61-69, 1999; P. Rockwell, et al., *Proc. Natl. Acad. Sci. USA*, 94, 6523-8, 1997; Hotoda, H., et al., *J. Med. Chem.*, 41, 3655-3663, 1998; Jing, N., et al., *J. Biol. Chem.*, 275, 3421, 2000). The antiviral mechanism of the oligonucleotides of the present invention seems to be similar to the antisense mechanism as reported previously in the point nonspecific reactions. However, anti-viral activity, safety and stability of oligonucleotides of the present invention have greatly improved by introducing the modified nucleotides containing 6-membered azasugar, which is the novelty of the present invention.

<5-2> Anti-viral activity of oligonucleotides within cells

To verify the anti-viral activity of oligonucleotides of the present invention was due to the inhibition of HIV-1 infection by acting outside of the cells, rather than their sequence-specific

inhibition by binding to the TAR RNA sequence, following experiments were conducted. Jurkat-tat cells ($2x10^6$) were transfected with 5 ㎍ of LTR-CAT plasmid, followed by addition of 2 ㎍ of oligonucleotides into medium(Fig. 9). On the other hand, Jurkat-tat cells were transfected with 5 ㎍ of LTR/CAT and 2 ㎍ of antisense oligonucleotides simultaneously (Fig. 10). Forty-eight hours after transfection, CAT (chloramphenicol acetyl transferase) activity was measured, which is proportional to the LTR/Tat activity. GenePorter™ transfectant kit (Gene Therapy System Inc. San Diego, CA, USA) was used for the transfection and experiment was performed according to the supplier's manual.

Forty-eight hours after transfection, cells were harvested and washed twice with PBS(4℃). After adding 1 ㎖ of TNB solution(40 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl), cell number was counted with hemocytometer. Three cycles of Freezing/thawing were repeated in ice/ethanol bath and 37℃ water bath to crush the cells. To inactivate the internal CAT activity, cell lysates were heated for 10 minutes in 60℃ water bath. CAT activity in each sample was measured by the method described in supplier's manual (Promega Co.)

As a result, CAT activity was not affected by

oligonucleotides when treated in the medium, which means oligonucleotides of the present invention could not inhibit LTR/Tat activity within the cells (Fig. 9). When the oligonucleotides were introduced into the cells by transfection, CAT activity was remarkably decreased due to the suppression of LTR/Tat activity by the oligonucleotides having no six-membered azasugar nucelotide (the SEQ. ID. NO: 4 and 13) or weakly inhibited by the oligonucleotide (the SEQ. ID. NO: 9) having six-membered azasugar nucleotides at both ends. On the contrary, LTR/Tat activity was not suppressed at all when oligonucleotides having more than three six-membered azasugar nucleotides (the SEQ. ID. NO: 2, 11 and 22) were introduced into cells by transfection while they showed strong anti-viral activity when added to the medium (Fig. 10). It means that the affinity of oligonucleotides to the target mRNA seems to be quite reduced by the presence of six-membered azasugars in the oligonucleotide, especially in proportion to the number of modified nucleotides in the oligonucelotides.

From these results, it was confirmed that the inhibitory effect of oligonucleotides containing six-membered azasugars on HIV-1 replication was not mediated by the sequence specific antisense mechanism against HIV gene, but rather mediated by inhibiting virus attachment on the cell surface. Owing to these characteristics, oligonucleotides of the present

invention were to have anti-viral activity against broad spectrum of HIV-1 strains including drug resistance mutant strains. As shown in the results of <Experimental Example 2>, oligonucleotides of the present invention (the SEQ. ID. NO: 20 and 22) showed potent anti-viral activity not only against laboratory strain and wild-type strain(IIIB), but also against SHIV89.6, an AZT-resistant and lethal recombinant virus. This also could be due to the fact that the oligonucleotides of the present invention block the virus infection by working on the outside of the cells. Hence, anti-viral activity of the oligonucleotides of the present invention against HIV-1 strains, resistant to reverse transcriptase inhibitor (Y181C, K103N, Y188H, L1001 and V106A) was determined. As a result, the oligonucleotides of the present invention showed strong anti-viral activity against these resistant strains.

Conclusively, unlike reverse transcriptase inhibitors or protease inhibitors, oligonucleotides of the present invention are expected to have anti-viral activity against wide variety of HIV-1 strains, thus they can be developed as therapeutic anti-AIDS agents.

<Experimental Example 6> Recovering effect on infected cells

Jurkat-Tat cells were infected with HXBc2/Δ tat as

described above experimental example <1-1>, and cultured in medium containing 10% FBS. The oligonucleotides of the present invention (0.5 μM) were added to the medium 5 days after infection, when many syncytia were formed. After 36 hours of treatment, the disappearance of syncytium formation was measured (Fig. 11).

As a result, in control group, large and active syncytium was observed on 7^{th} day. But, in groups treated with oligonucleotides of the SEQ. ID. NO: 2, 17, 20 and 22, syncytium formation was significantly decreased. Especially, when oligonucleotide of the SEQ. ID. NO: 22 was treated, most of syncytium formed disappear and the cells were returned to normal condition. However, when oligonucleotide having P=S backbone without 6-membered azasugars (represented by the SEQ. ID. NO: 4) was treated, syncytium formation was more active than that of control.

As shown in the above results, oligonucleotides containing 6-membered azasugars can suppress virus infection in the initial stage as well as virus propagation, which has shown by the inhibitory effect on syncytium formation mediated by cell-to-cell fusion through gp120-CD4 binding. Therefore, if oligonucleotides of the present invention were given to AIDS patients, they would block the virus infection to the normal cells and suppress syncytium progression,

resulting in recovering of the AIDS patients.

<Experimental Example 7> Anti-viral activity of oligonucleotides against other viruses than HIV-1

In order to confirm whether the oligonucleotides of the present invention show anti-viral activity against other viruses than HIV-1, following experiment was performed using antisense oligonucleotides (the SEQ. ID. NO: 6 and 7, containing six-membered azasugars) corresponding to the TAR region of SIV virus (AIDS Research and Reference Reagent Program, NIH, USA) and antisense oligonucleotide (the SEQ. ID. NO: 8) to the IRES (internal ribosomal entry site) sequence of poliovirus.

<7-1> Anti-viral activity of oligonucleotides against SIV

While culturing CEMx174 cells in RPMI medium supplemented with 10% FBS, SIV was infected (0.01 MOI) thereto as described above experimental example <1-1>. After infection, oligonucleotides of the present invention were added into the wells (final conc. 0.5 μM). Supernatant was harvested every other day, and reverse transcriptase activity was measured as described in experimental example <1-2> to determine the titer of virus (Fig. 12).

As a result, none of the oligonucleotides of the present invention could inhibit SIV replication. This means that the anti-viral activity of oligonucleotides containing six-membered azasugars of the present invention is HIV-1 specific. While SIV has a similar gene structure with HIV-1, the sequence homology between SIV and HIV-1 genes is about 50%, and host specificity is also different, therefore human beings are not infected with SIV. Considering that the host-specificity is different between SIV and HIV-1, it is explained that the oligonucleotides of the present invention exert anti-viral activity through the mechanism of blocking HIV-1 infection specifically by binding to receptor or envelope proteins that are essential for the virus attachment.

<7-2> Anti-viral activity of oligonucleotides against poliovirus

HeLa cells cultured by mono-layer on 30 ㎜ culture dish, were infected with poliovirus(Sabin type I, obtained from Dr. E. Wimmer, State University of New York, USA) for 1 hour (0.1 MOI) followed by washing twice with PBS to remove uninfected virus. DMEM medium(supplemented with 10% FBS, GIBCO/BRL) containing 1 μM oligonucleotides was added thereto for further culturing. While culturing, supernatants were obtained every 3 hours and the amount of virus was measured by

$TCID_{50}$ assay (Fig. 13).

As a result, none of the oligonucleotides of the present invention affect on poliovirus replication regardless of sequence specificity. This result suggests that the oligonucleotides could not exert anti-viral activity within the cells since they could not get into the cells without special delivery system such as liposome, and also had no effect on poliovirus receptor highly expressed on the cell surfaces.

Inventors' experiments have shown that oligonucleotides of the present invention inhibit the HIV-1 replication not by intracellular antisense mechanism but by blocking the cell surface receptor as reported previously (P. Hawley, et al., *Antisense Nucleic Acid Drug Dev.*, 9, 61-69, 1999; P. Rockwell, et al., *Proc. Natl. Acad. Sci. USA*, 94, 6523-8, 1997), or by blocking V3 region of HIV-1 envelope proteins (J. Suzuki, et al., *Nucleic Acids Symp. Ser.*, 42, 227-228, 1999; J. R. Wyatt, et al., *Proc. Natl. Acad. Sci. USA*, 91, 1356-1360, 1994). Nevertheless, as shown in the results of <7-1> and <7-2>, the oligonucleotides of the present invention had no influence on the replication of other viruses than HIV-1, suggesting that the treatment of the oligonucleotides of the present invention did not affect on the function of other cell

surface molecules.

<Experimental Example 8> Serum effects on the anti-viral activity of oligonucleotides

Oligonucleotides of the present invention should have an anti-viral activity in the presence of high concentration of serum when they are administered to human beings. To investigate whether the anti-viral activity of oligonucleotides is affected by the concentrations or types of serum, anti-viral activities of oligonucleotides in serum-free medium (Opti-MEM, GIBCO/BRL), RPMI-1640 medium (GIBCO/BRL) supplemented with 10% FBS, 10% horse serum, human serum and different concentrations of FBS were measured. Jurkat-tat cells were infected with HIV-1/Δ tat as described in the experimental example <1-1>, followed by adding 0.1 μ M of oligonucleotides. And then, the date was checked when the syncytium formation was first observed and the results were summarized in Table 5.

As shown in Table 5, in a serum-free medium (Opti-MEM), oligonucleotides (represented by the SEQ. ID. NO: 2, 11, 17, 22 and 29) of the present invention showed high anti-viral activity except the oligonucleotide of the SEQ. ID. NO: 4, where the syncytium formation was first observed 8 days after infection. However, when serum was added, anti-viral activity was rather

decreased, resulting in that the syncytium formation was observed 2-3 days earlier than the serum-free condition. Especially, the oligonucleotide of the SEQ. ID. NO: 2 was most affected by serum, and the anti-viral activity of the oligonucleotide of the SEQ. ID. NO: 22 was the least affected in the presence of serum. The inhibitory effect of serum on anti-viral activity of oligonucleotides was independent of serum types or concentrations. Even low concentration of serum affected on the anti-viral activity of oligonucleotides once serum was added. That was probably because even small amounts of serum would be enough to bind to oligonucleotides to the point of saturation. Thus, the high concentration of serum *in vivo* would not reduce the anti-viral activity of oligonucleotides of the present invention further. It is noteworthy that oligonucleotides of the SEQ. ID. NO: 22 and 29 keep their anti-viral activity without being influenced by the presence of serum, type or concentration of serum.

<Experimental Example 9> Acute toxicity in mice by intravenous administration

The following experiments were performed to see if the antisense oligonucleotides of the present invention have acute toxicity in mice.

5-week old SPF ICR line mice were used for acute toxicity test. Each oligonucleotide of the present

invention was dissolved in PBS and injected intravenously at the dosage of 100 mg/kg to 3 mice per group. Death, clinical symptoms, and weight change in mice were observed, hematological and biochemical tests in blood were performed, and any abnormal signs in chest and abdomen were checked by naked eyes after autopsy, in which no specific clinical symptoms, weight change, death, nor changes in hematological and biochemical tests were resulted. These results indicate that the oligonucleotides of the present invention are considered to be safe by intravenous administration into mice up to 100 mg/kg.

INDUSTRIAL APPLICABILITY

As shown above, oligonucleotides of the present invention, containing six-membered azasugar instead of five-membered ribose for 3 to 5 nucleotides, and consisting of phosphorothioate (P=S) backbone, have strong anti-HIV-1 activity by working outside of the cells and long-term stability to nucleases. Therefore, oligonucleotides of the present invention can be used as an effective anti-AIDS agent.

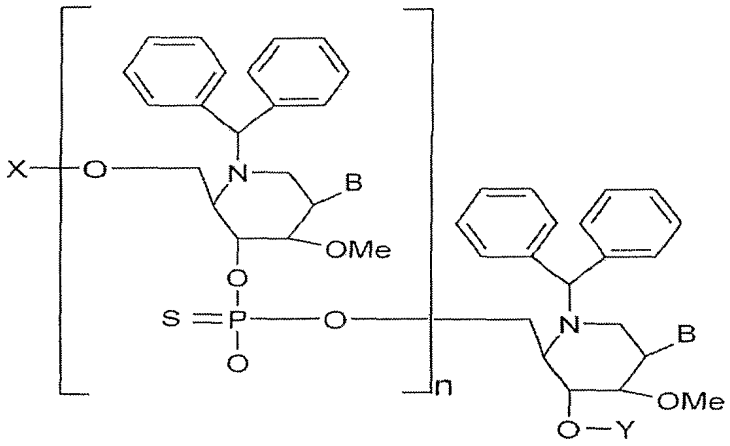
Those skilled in the art will appreciate that the

conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is claimed is

1. Oligonucleotides having the <Chemical Formula 1> structure shown below whose five-membered ribose, a natural nucleotide sugar, is substituted with six-membered azasugar, and consisting of phosphorothioate (P=S) backbone.

<Chemical Formula 1>



In the above <Chemical Formula 1>,

n = 0 ~ 30,

1) B is a wild-type or a modified nucleobase with or without a protecting group,

2) X is hydrogen, hydroxyl protecting group, conjugate group or oligonucleotide,

3) Y is hydrogen, phosphate, activated phosphate, activated phosphite, solid support, conjugate group or

oligonucleotide.

2. Chimeric oligonucleotides containing phosphodiester or phosphorothioate oligonucleotides in the center, and having oligonucleotides of claim 1 in the flank.

3. The oligonucleotides as set forth in claim 1, wherein the oligonucleotide is selected from the group consisting of oligonucleotides of the SEQ. ID. NO: 2, 3, 11, 17, 18, 19, 20, 21, 22, 23 and 29.

4. A pharmaceutical composition used for the treatment of AIDS containing the oligonucleotides of claim 1.

5. The pharmaceutical composition used for the treatment of AIDS containing the chimeric oligonucleotides of claim 2.

Figures

FIG. 1

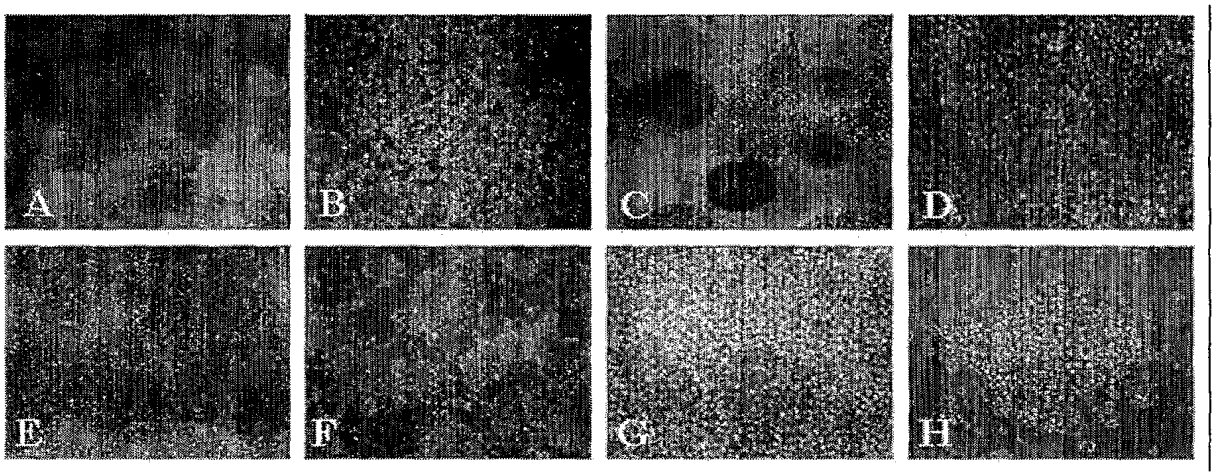


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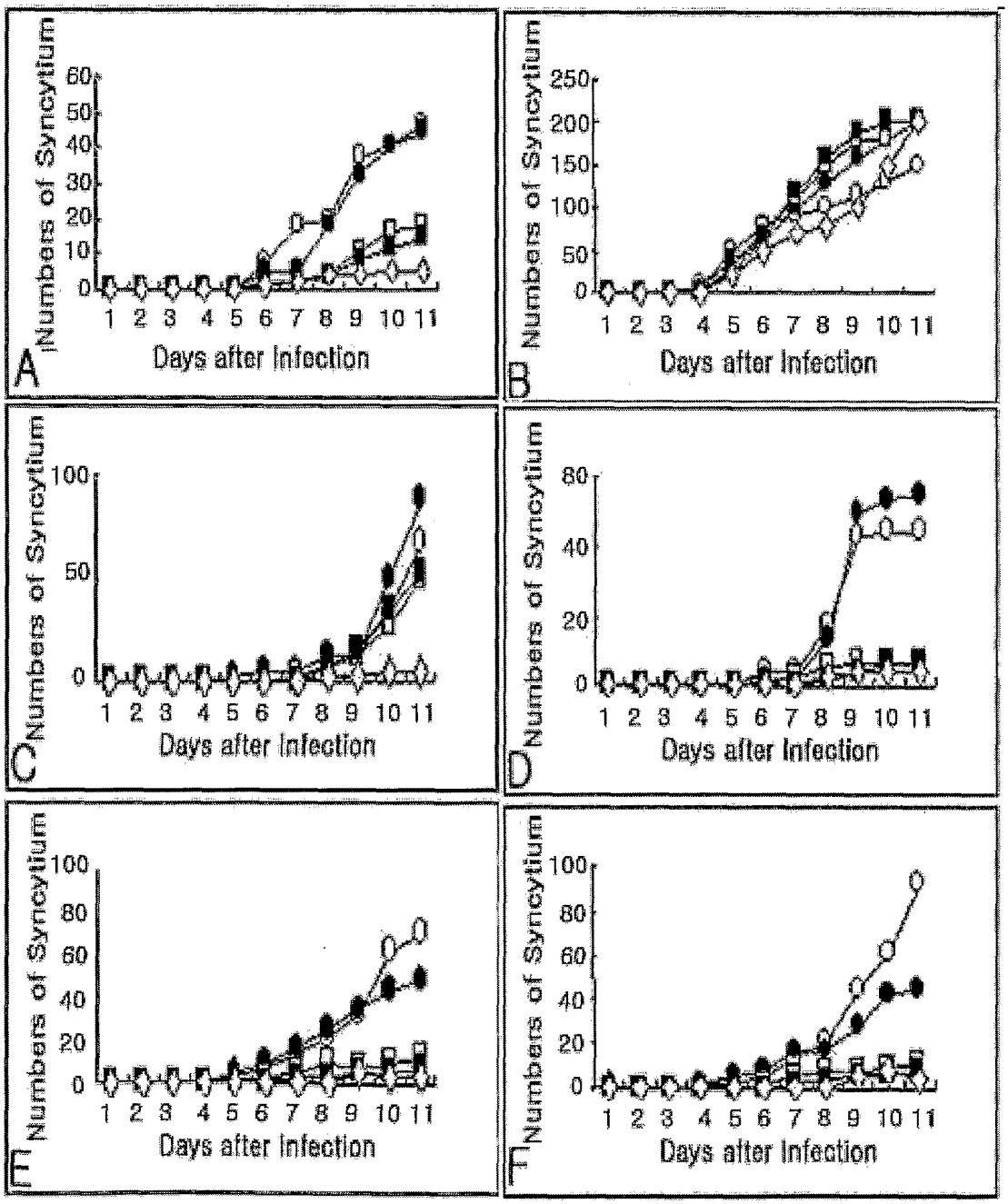
FIG.2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50
40
30
20
10
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Days after Infection
B
Numbers of Syncytium
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11
Days after Infection
C
Numbers of Syncytium
100
50
0
1 2 3 4 5 6 7 8 9 10 11
Days after Infection
D
Numbers of Syncytium
80
40
20
0
1 2 3 4 5 6 7 8 9 10 11
Days after Infection
E
Numbers of Syncytium
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11
Days after Infection
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Numbers of Syncytium
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60
40
20
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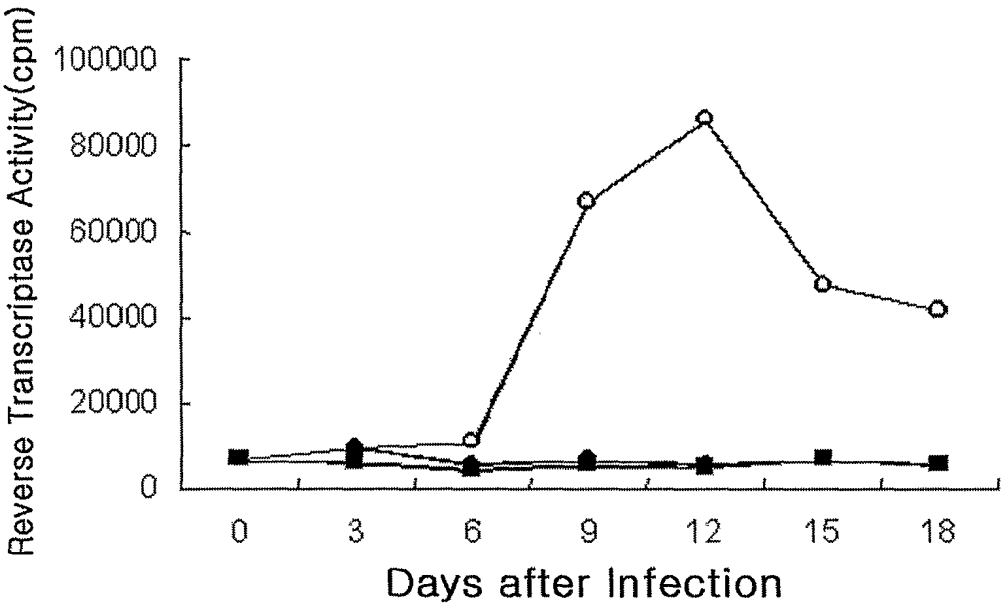
1 2 3 4 5 6 7 8 9 10 11
Days after Infection

FIG. 3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60000
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20000
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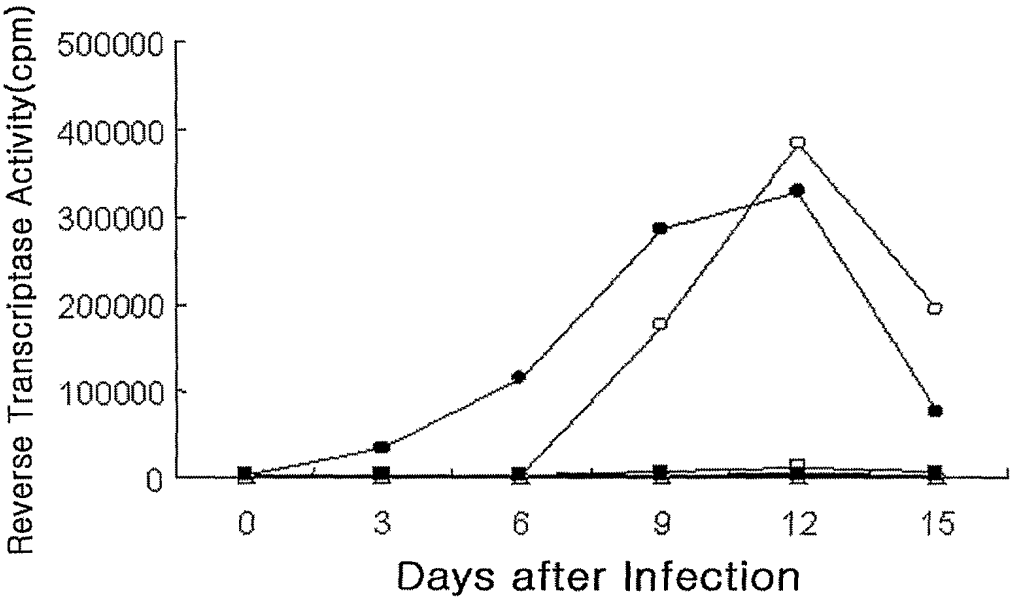
0
3
6
9
12
15
18
Days after Infection

FIG. 4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6
9
12
15
Days after Infection

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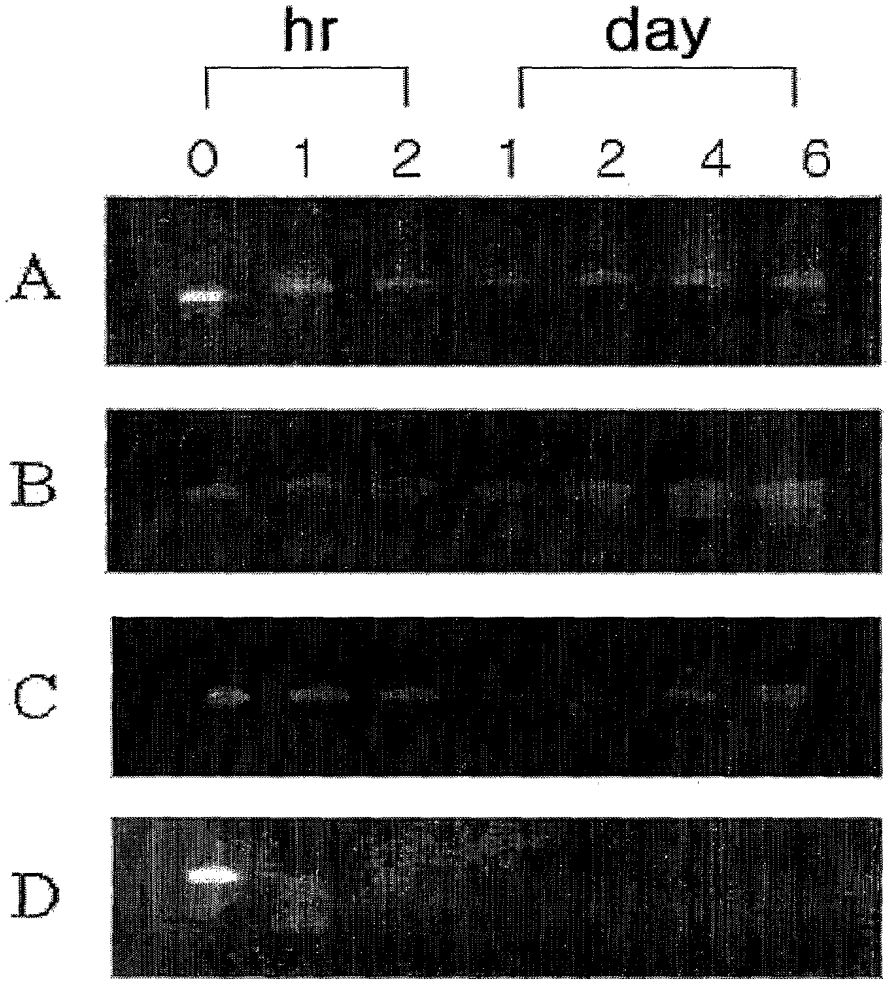

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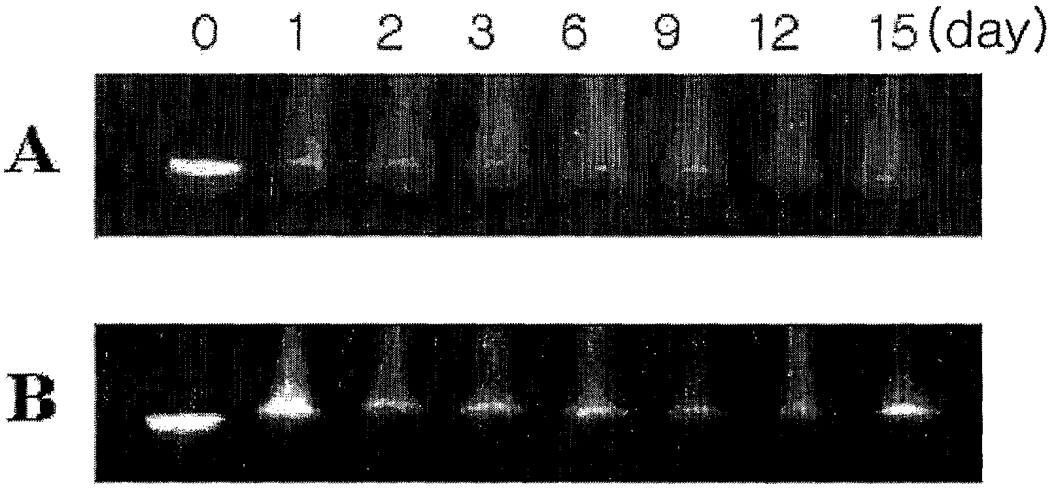
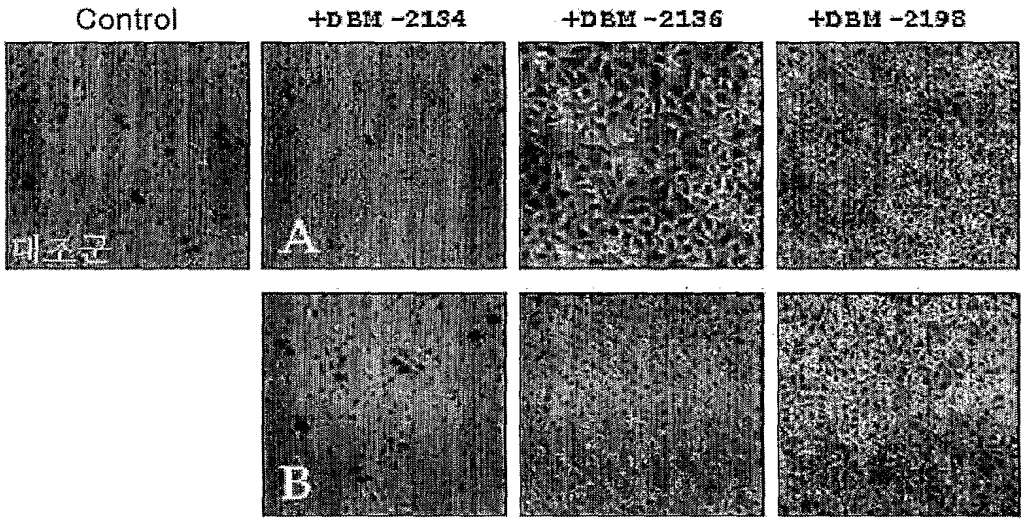


FIG. 7



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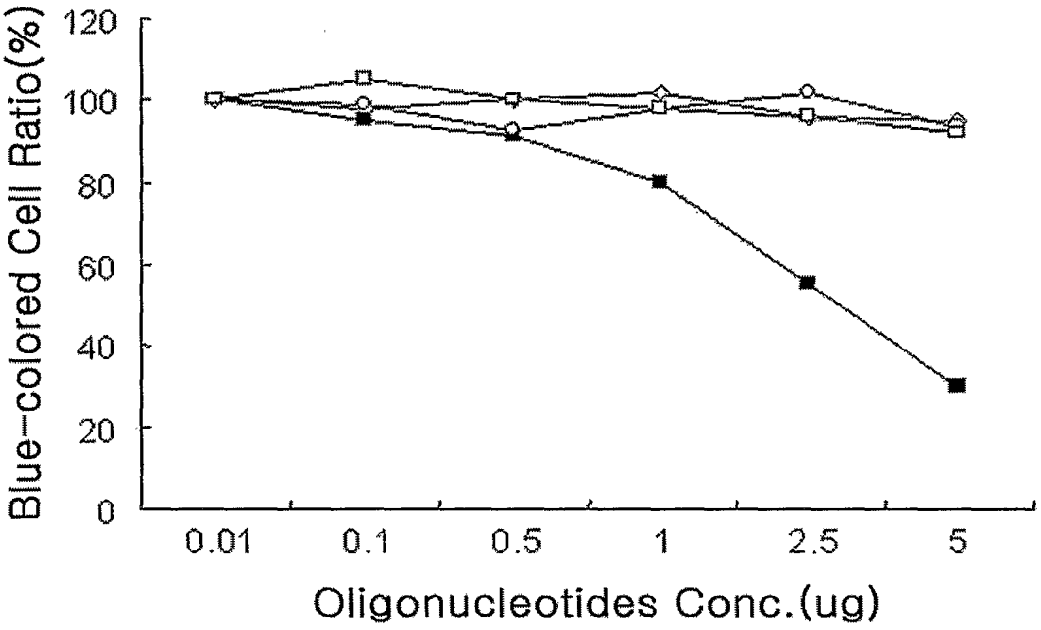
Blue-colored Cell Ratio(%)
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40
20
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0.01
0.1
0.5
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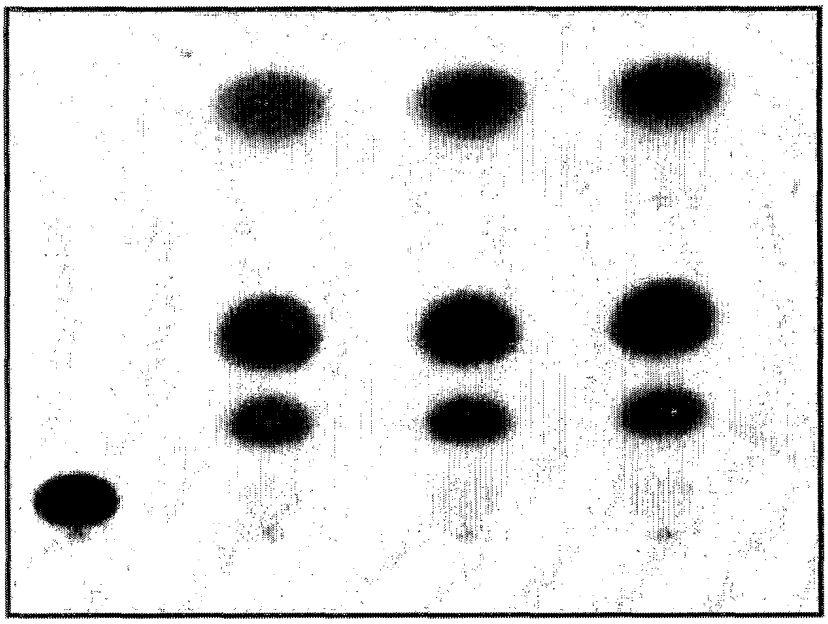
2.5
5
Oligonucleotides Conc.(ug)

FIG. 9



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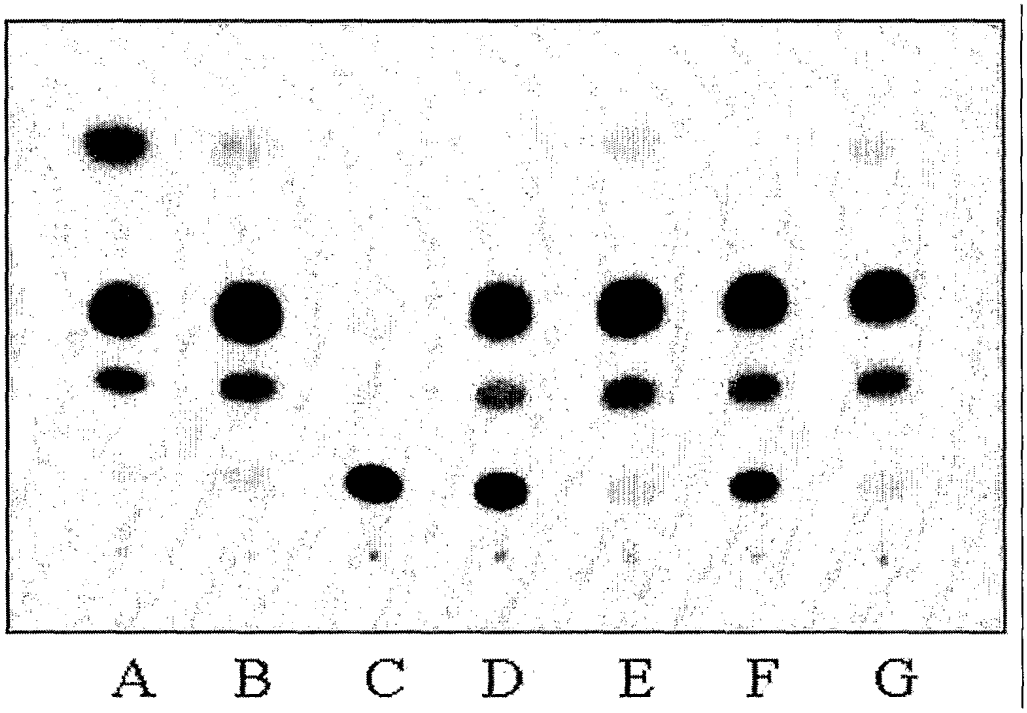

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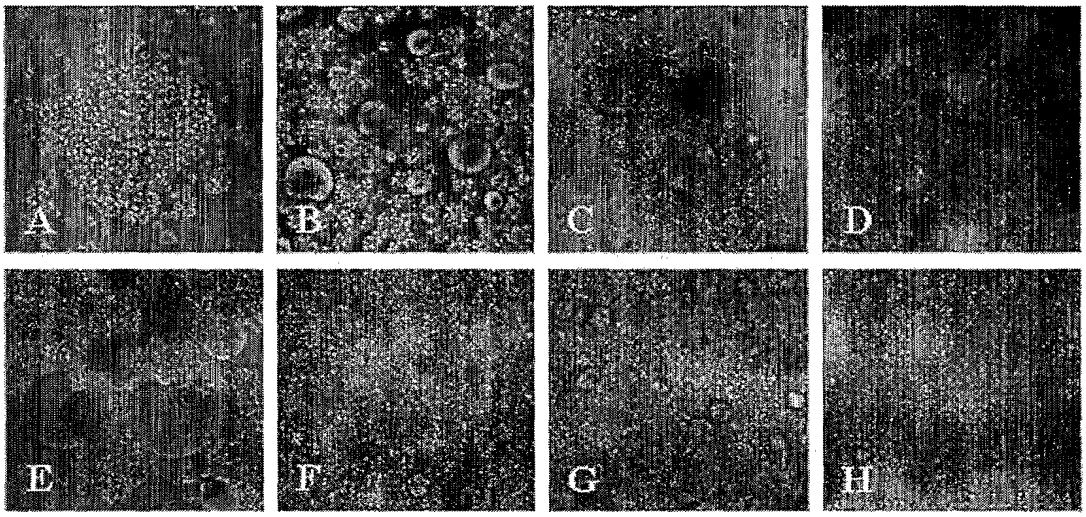

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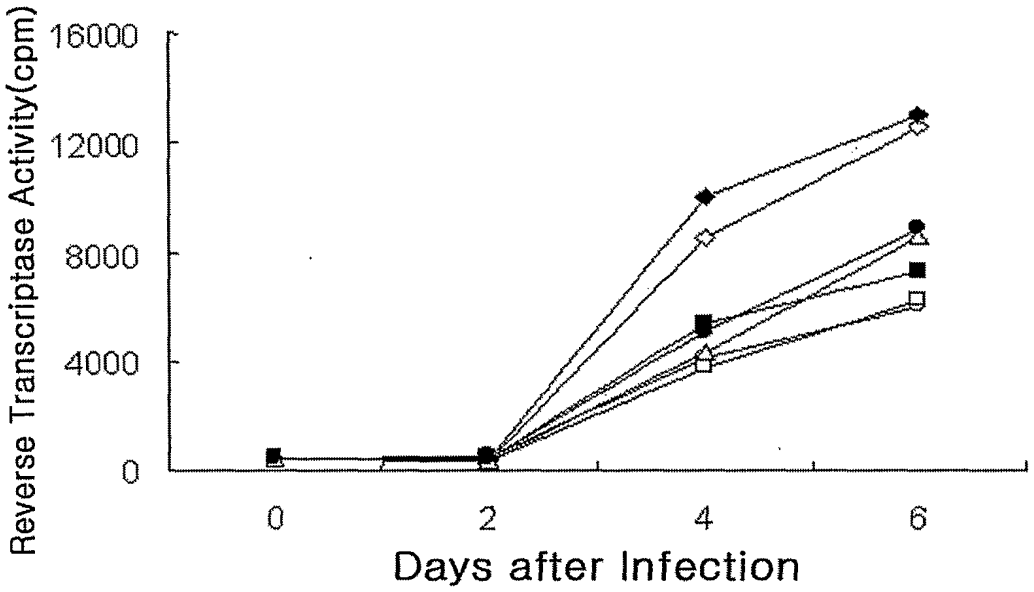
FIG. 12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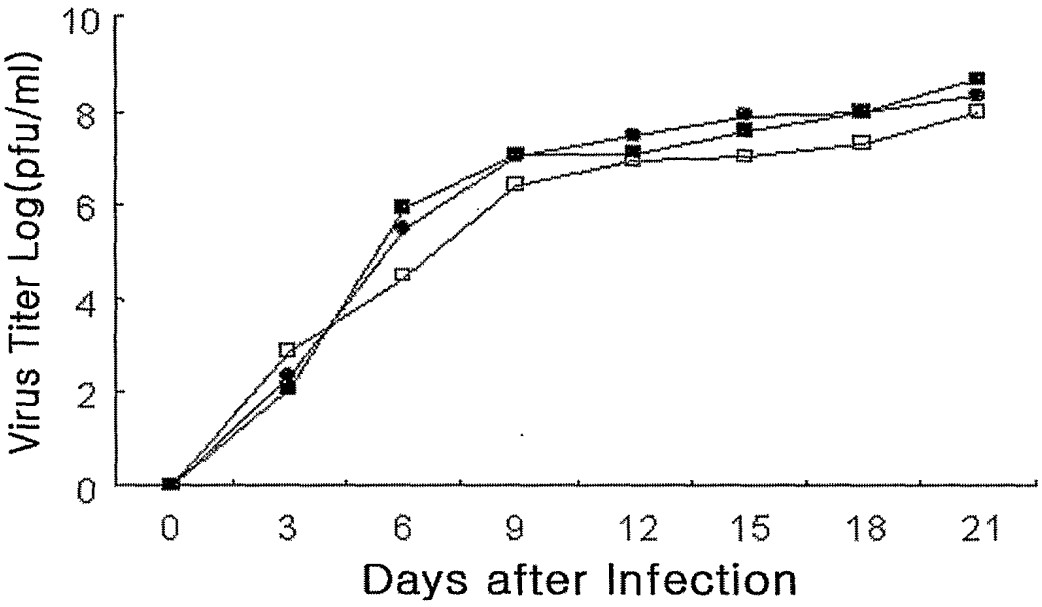
2
4
6
Days after Infection

FIG. 13


Virus Titer Log(pfu/ml)
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8
6
4
2
0
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3
6
9
12
15
18
21
Days after Infection

SEQUENCE LISTING

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